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A SIMPLE METHOD OF IDENTIFYING *STREPTOCOCCUS AGALACTIAE*

By

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INTRODUCTION

This paper describes a method of identifying *Str. agalactiae* by its reactions in a medium containing sodium hippurate, aesculin and arginine mono-hydrochloride. It is generally well-known that *Str. agalactiae* hydrolyses sodium hippurate and arginine mono-hydrochloride and does not split aesculin, but the high specificity of these characteristics as a means of identifying it has not, to the author's knowledge, been stressed before. As most of the readers interested in this article will be familiar with the literature, only covering references will be given All streptococci are referred to by their species name without the generic prefix.

Ayers and Rupp (1922) showed that 44 strains of β haemolytic streptococci from the bovine udder could be distinguished from 33 β haemolytic streptococci of human origin by their reduction of sodium hippurate. They stressed that the test should be used for β haemolytic organisms only, but it has since been widely applied in the identification of α and non-haemolytic strains, particularly *agalactiae*. Ayers *et al.* (1921) studied the production of ammonia and carbon dioxide by streptococci and concluded that the formation of these substances from peptone represented "fundamental differences in the physiology of the streptococci." Ayers and his associates in subsequent papers (Ayers and Mudge, 1922, 1923; Ayers *et al.*, 1924) applied the ammonia test in the classification of these organisms from various sources. They endeavoured unsuccessfully to trace the source of the ammonia and, suspecting that it came from the amino acids or acid amides, tested eight of the former and two of the latter. Unfortunately arginine was not among them.

Hills (1940) further studied the source of the ammonia produced from peptone by streptococci by testing the individual amino acids and found that arginine was the principal one. Niven *et al.* (1942) examined the production of ammonia from arginine mono-hydrochloride by known strains of streptococci and confirmed Hills's findings; they showed that the pathogenic streptococci as well as *faecalis* and *lactis* hydrolyse this substance, while *bovis, salivarius, equinus* and *cremoris* do not. These workers devised a medium which is a really valuable aid in the study of streptococci; it is easily prepared, streptococci grow well in it, the reading of the results is simple and doubtful results are rare.

METHODS

The author has just completed a comprehensive study of the bacteriology of the bovine udder. In this work 336 streptococci

were tested for their action on sucrose, lactose, mannite, trehalose, salicin, raffinose, inulin, maltose, arabinose, glycerol, sodium hippurate, aesculin and litmus milk. Of these strains the only ones which reduced the hippurate and did not split aesculin were all the *agalactiae* and two *bovis*. One hundred and forty-one of these cultures were further tested as follows : growth in methylene blue milk at concentrations of 1 in 1,000, 1 in 5,000, 1 in 10,000 and 1 in 20,000 ; final *p*H in 0·5 per cent. glucose broth ; hydrolysis of arginine mono-hydrochloride ; fermentation of starch ; growth on 10 per cent. and 40 per cent. bile in sheep blood agar ; production of gas from 5 per cent. glucose broth ; liquefaction of gelatine ; growth at *p*H 9·6 and 6·5 per cent. in sodium chloride broths ; resistance to 55° C. and 60° C. for 30 minutes ; growth at 10° C. and 45° C. ; production of catalase ; solubility in sodium desoxycholate, 1 in 500 ; reaction of groups A, B, C, D, F, G, H, K, L and N to streptococcal antisera by the precipitin technique (Lancefield, 1933). It is not proposed to give details of all the results here as this will be done in another paper to be published later. However, it can be said that the 141 cultures included representative numbers of *agalactiae, dysgalactiae, uberis, faecalis, lactis, bovis, salivarius, cremoris,* groups G and K and a small indeterminate group which did not fit in with any of the established species. The primary object of this work was the certain identification of species, but as so many different species had been isolated and they had been so extensively examined, a useful by-product was the determination of the least number of tests required to differentiate the principal pathogens from the extraneous bacteria and from one another. It is to be noted that the Lancefield technique was by no means perfect in this respect, even with group B. It was found that the only streptococci which were positive hippurate, negative aesculin and positive ammonia were *agalactiae*. (These latter tests were done in separate media.)

Hippurate and aesculin are generally used in the same medium in the examination of udder streptococci in this country. It was, therefore, decided to test the action of a variety of known streptococci on sodium hippurate, aesculin and arginine mono-hydrochloride in one composite medium for, if the various reactions were reproduced a considerable saving of time could be achieved in handling large numbers of cultures. The hippurate-aesculin-ammonia test will be called HAA. Three strains each of streptococci having the following reactions were tested: + − + (*agalactiae*), − − + (*dysgalactiae*), + + + (*uberis*), − + − (*bovis*), − + + (group G), − − − (*salivarius*) ; a single strain of *bovis* having the reaction + + − was also tested. The reaction of these organisms in the composite medium were the same as when done in separate tubes.

The composite medium was that of Niven *et al.* (1942) plus 0·1 per cent. aesculin and 2 per cent. sodium hippurate. It had the following composition:

	per cent.
Difco yeast extract	0·5
Difco tryptose peptone *	0·5
Di-potassium phosphate	0·2
Glucose	0·05
Arginine mono-hydrochloride * ...	0·3
Aesculin	0·1
Sodium hippurate	2·0

*p*H 7·4

This medium was incubated for 48 hours, more than 1 c.c. removed with a sterile pipette, the excess run into one small test tube and the remaining 1 c.c. into another ; to the latter was added 0·8 c.c. of a solution of 12 per cent. ferric chloride in 2·5 per cent. HCl and the tube shaken immediately. Any opacity was taken as reduction but *agalactiae* has always given a heavy precipitate ; splitting of the aesculin was shown by a dark green coloration. With organisms that had no action on either, the medium remained unchanged. A few drops of Nessler's reagent were added to the first tube and formation of ammonia evidenced by the production of a heavy, deep orange precipitate.

Two per cent. hippurate was used since in the early part of the author's work on udder streptococci some obvious *agalactiae* were found which gave only very slight reduction in the 1 per cent. concentration. The opinion was formed that in these cases full reduction of the hippurate did occur but that the amount of ferric chloride required to redissolve the protein was also redissolving the benzoate. Plastridge and Hartsell (1937) also report some indefinite results with 1 per cent. hippurate and found that " it was usually hydrolysed " by *agalactiae* and that " the results obtained with cultures which required the use of beef infusion in this medium were not always satisfactory " Stableforth (1937) records two out of 42 hippurate negative *agalactiae* and Stewart (1937) one out of 52. In the author's experience *agalactiae* appears to attack hippurate actively and when the concentration is 2 per cent. a heavy curd settles on the addition of the ferric chloride.

The HAA medium was given an extended trial in the weekly milk examinations from four herds affected with mastitis. The routine was as follows: Streptococcus colonies were replated on quarter plates and isolated colonies cut out and dropped into 0·5 per cent. glucose broth. These were incubated overnight, seeded into Hartley's broth and spun down ; if autoagglutinable or granular, one drop of phenolphthalein and two drops of trypsin were added and *p*H brought to between 8·4 and 9·0, *i.e.*, when suspension turned pink. The organisms were then digested overnight.* After which a small drop was placed on a slide and mixed with some

* Niven *et al.* used bactotryptone for their arginine medium.

agalactiae antiserum which was known to agglutinate all the common types. The Hartley's broth, after overnight incubation, was further seeded into the HAA medium and kept for reference until identification of the organism was complete.

RESULTS

Well over 1,000 streptococci from routine milk samples have been examined in this medium in combination with the slide agglutination test and the Lancefield precipitation test, and in most instances with the four carbohydrates trehalose, salicin, raffinose and inulin. The basic part of the examination for the identification of *agalactiae* has been the slide agglutination test versus a mixed type serum and reaction in the HAA medium. Those that were HAA + – + and negative in the serum or autoagglutinable were examined by the Lancefield precipitin test and the slide agglutination test against a number of sera. In only one instance was a culture found which was HAA + – + and not *agalactiae*. This culture was examined personally by the author and gave weak but definite reduction of hippurate after 48 hours and five days, and also fermented raffinose. The reaction was easily distinguishable from that given by all the agalactiae cultures. Two others were also found which were HAA + – + and *agalactiae* by slide agglutination but which fermented raffinose ; in this case there seems little doubt that the raffinose fermentation was due to contamination ; both cultures had been discarded before the discrepancies were discovered.

Nine hundred and seventy-nine streptococci were examined from the four mastitis herds and the following data are given for those which were suspected of being *agalactiae* on one or more reactions.

Agglutination positive, HAA + – +	...	223
Agglutination negative, HAA + – +	...	27
Agglutination positive, HAA negative ...	...	44
Autoagglutinable, HAA + – + ...	...	15

Of the HAA positives, the 27 agglutination negatives and the 15 autoagglutinables were confirmed as *agalactiae* by either the precipitation test or the slide agglutination test against other Group B sera. One transfer in glucose broth followed by tryptic digestion was usually sufficient to make stable suspensions of the autoagglutinables. The 44 agglutination positive, HAA negative organisms were mostly *uberis* and some gave good reactions with several Group B antisera by precipitin test.

DISCUSSION

In the present work the reactions of *agalactiae* in a medium containing sodium hippurate, aesculin and arginine mono-hydro-

* This procedure is not original. It was demonstrated by Mr. W. R. Maxted, of the Emergency Public Health Laboratory, Ravenscourt Square, London, as a means of overcoming auto-agglutinability in streptococcal suspensions.

chloride have proved highly specific as a means of differentiating this organism from other udder streptococci. In only one instance was a streptococcus found which hydrolysed hippurate and arginine mono-hydrochloride and had no action on aesculin and yet was not *agalactiae ;* this reduced hippurate to a much lesser degree than all the *agalactiae* strains and also fermented raffinose slowly.

Provided the organism is a streptococcus in pure culture the HAA test appears to give higher specificity than either the group precipitin or agglutination tests but is probably not quite so water-tight as the type precipitin and agglutination tests. One false positive in over 1,000 cultures is higher than this laboratory could claim for the serological methods and seems sufficient justification for a more extended application of the test by other workers. It is not recommended that it should replace the serological methods but, in the author's opinion, any culture that is not HAA + – + is not *agalactiae,* irrespective of its serological reactions. As *agalactiae* strongly hydrolyses both hippurate and arginine and never splits aesculin, the test should give no false negative reactions.

There are also certain practicable advantages over the serological diagnostic methods ; the medium is easy to prepare, the reactions are easy to read, no difficult techniques or elements of personal interpretation or fine judgment are involved and, finally, it is time-saving.

As Sherman (1937) and Niven *et al.* (1942) have shown that any system of examination of streptococci other than β haemolysis which does not include the ammonia test, is useless, the present medium in conjunction with either the serological or biochemical methods forms a good primary basis for the identification of the pathogenic udder streptococci. In the case of milk streptococci all ammonia negatives and ammonia positives that do not reduce hippurate but split aesculin can be discarded, unless the latter are strongly haemolytic. Strains giving other reactions can be inoculated into trehalose, salicin, raffinose and inulin. The following table illustrates the reactions of the three main pathogens :

	Trehalose	*Salicin*	*Raffinose*	*Inulin*	*Hippurate*	*Aesculin*	NH_3
Agalactiae	+	+	–	–	+	–	+
Dysgalactiae	+	–	–	–	–	–	+
Uberis	+	+	–	+	+	+	+

Agalactiae usually ferments trehalose or salicin, or both, while the fermentation of inulin without the fermentation of raffinose is so rare a characteristic among streptococci as to be almost specific for *uberis.* This series of biochemical tests will identify certainly any of the three main pathogens should, for any reason, the sera reactions of the first two be at fault. The other tests given earlier in this paper add nothing of further differential value. The carbohydrates tests for *dysgalactiae* can be eliminated by testing it either by slide agglutination or the Lancefield precipitin method against a

group C serum which is known to be specific. Unless the group C serum contains no non-specific antibody for other streptococci, either by its previous removal or careful selection of the immunising strains, it is unwise to depend on the serological methods alone.

For the routine identification of the udder pathogens the present practice of this laboratory is to depend on HAA and slide agglutination for *agalactiae*, HAA and the precipitin test against an " animal pyogenes " group C serum for *dysgalactiae* and HAA and fermentation of inulin without fermentation of raffinose for *uberis*. In the author's opinion this is the absolute minimum upon which certain identification of species can be based. Organisms which are HAA + − + but are auto- or inagglutinable are nevertheless regarded as *agalactiae* unless some very obvious discrepancies occur. Strongly haemolytic organisms are always examined further by the usual bio-chemical and serological methods which need not be given here.

SUMMARY

Over a thousand strains of milk streptococci have been examined for their reactions in a medium containing sodium hippurate, aesculin and arginine mono-hydrochloride. With one exception all organisms which hydrolysed sodium hippurate and arginine and failed to split aesculin, were *Str. agalactiae.*

Since the data in this paper were collected the HAA method has been in daily use in this laboratory for three months ; the findings throughout have been as above with the exception of two strains of *Str. dysgalactiae* from the same cow which have persistently failed to grow in the medium. These are the only streptococci encountered which have failed in this respect.

ACKNOWLEDGMENTS

The author wishes to acknowledge his gratitude to Mr. W. R. Maxted of the Emergency Public Health Laboratory, Ravenscourt Square, London, W.6, for much helpful advice on the serology of streptococci, to Miss Marjory White who did the work on the routine milk samples, to the Director of the Institute, Dr. J. Russell Greig and to Dr. A. D. McEwen, for their constant interest.

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AN EPIZOOTIC OF INFLUENZA IN A FERRET COLONY

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INTRODUCTION

Wilson Smith *et al.* (1933) in their classical experiments on the isolation of the virus of influenza, showed that the disease was transmitted experimentally to ferrets by the intranasal inoculation of throat washings from cases of human influenza. Stuart-Harris *et al.* (1938) demonstrated that infection was readily transmitted amongst ferrets if the animals were kept in the same cubicle or in close contact one with another. Wilson Smith and Stuart-Harris (1936) also showed that it was possible for a ferret to transmit the infection back to man. Andrewes and Glover (1941), in more detailed experiments on cross-infection with influenza virus A in ferrets, showed the importance of " droplet nuclei " and other very small particles in the spread of infection from one ferret to another. These latter experiments stressed the need for complete isolation of individual animals prior to use in experimental work with the influenza viruses.

Records of influenza epizootics occurring amongst ferrets in the natural state are rare and as far as we can ascertain only one has previously been described (Fisher and Scott, 1944).

It does not necessarily follow, however, that the disease does not occur in ferrets since animals are generally kept in very small groups and might contract the disease without its being recognised. There are very few establishments in which ferrets are maintained in sufficient numbers to favour the development of an epizootic. This article describes an outbreak of influenza in two ferret colonies during February, 1947, at a time when there were many localised outbreaks of human influenza occurring throughout the country (Dudgeon *et al.*, 1948) and reaching a peak at the end of the first week in February.

THE COURSE OF THE OUTBREAK

Both colonies were situated at the Wellcome Veterinary Research Station, Frant, Sussex. The animals were bred for research work in connection with canine distemper and were, therefore, maintained under conditions which would prevent exposure to the virus of that disease. The colonies were entirely separate as regards maintenance and attendance, and were about one mile apart.

In colony A, the animals occupied a single isolated building which was divided into a series of courts, 12 ft. by 7 ft. or 7 ft. by 7 ft., on each side of a central passage-way. A brick wall, 4 ft. high served to separate the courts, the front of each being closed by a piece of metal 2 ft. 6 in. high. The colony comprised 194 animals (59 males and 135 females) which were distributed in groups of about 20 to each court, the males and females being kept separate. In colony B the animals occupied a single building which was divided into a number of fairly small rooms 15 ft. by 7½ ft. without a complete door, the rooms being closed by a sheet of metal 2 ft. 6 in. high. Only the attendants were allowed into the colonies and they were required to change into rubber boots and pass through a foot-bath of disinfectant in a double doored entrance room before entering the building.

The animals in both colonies first showed signs of an infection of the upper respiratory tract on February 3rd, 1947. The clinical signs were similar in all affected animals. There was a muco-serous nasal discharge which produced crusts around the nostrils: in addition the conjunctiva was inflamed and the muco-purulent exudate often caused the eyelids to stick together. There was frequent sneezing. In addition to these symptoms, a few of the infected animals developed a unilateral otitis with a purulent discharge. The sick animals were lethargic but their appetite was maintained with an avidity for milk or water. In some instances rectal temperatures were taken, and readings up to 105° F. were recorded.

The disease ran its course in individual animals in about seven days.

During January, five of the nine personnel of the animal breeding unit of which ferret colony B formed a part, were granted sick-leave because of influenza. Attendant K who was in charge of colony B was away from work during January 16th to 19th with influenza-like symptoms although he did not seek medical advice. Attendant G who was in charge of ferret colony A had an attack of influenza during the last few days of January, but did not ask for sick-leave.

In colony A the disease had disappeared by February 22nd and as far as can be ascertained, almost all the ferrets had been infected. During the period there were four deaths (4 ♀♀) of which only two were referable to the influenza-like disease, but neither of these animals showed any consolidation of the lungs. In colony B the epizootic was slightly more prolonged, lasting until February 28th. During this period 38 males which were confined to one room were affected. It was noticeable that a number of the ferrets did not show any obvious symptoms. There were four deaths during the period (1 ♂, 3 ♀♀) in the whole colony.

LABORATORY EXAMINATION

Post-mortem examination of ferrets which died during this period showed no macroscopic lesions other than congestion of the upper respiratory tract with a serous exudate from the mucous membrane of the nasal chambers and the pharynx. Bacteriological examination of all organs proved negative for pathogenic bacteria except for a haemolytic streptococcus which was isolated from the lungs and which proved to be of Lancefield's Group C. There was no pulmonary consolidation or other macroscopic lesions in the lungs. Blood was obtained by cardiac puncture from several ferrets in each colony and by venepuncture in the case of the attendants ; the serum was separated and inactivated at 56° C. for 20 mins. Each serum specimen was tested for influenza antibody titre by the red cell agglutination method described by Salk (1944). The antigens used were the standard P.R.8 (A), and two current A viruses (Kunz and Bar, 1947). The results are shown in Table I. A few sera were

TABLE I

RECORDS OF HAEMAGGLUTINATION TESTS

Source of sera	*Serum number*	*Titres against influenza virus.**		
		P.R.8	*Bar (A)* 1947	*Kunz (A)* 1947
Colony A	1	64	N.T.†	2,048
	2	64	4,090	2,048
	3	40	640	640
	4	64	1,024	2,048
	11	32	4,096	2,048
	12	32	2,048	1,024
	13	16	2,048	2,048
	Attendant G	512	1,024	1,024
Colony B	5	N.T.†	2,048	2,048
(Animals which showed symptoms)	6	32	2,048	2,048
	7	64	1,024	4,096
	8	64	2,048	2,048
	9	64	1,024	1,024
	Attendant K	512	1,024	2,048
Colony B	15	32	4,096	2,048
(Animals which did not show symptoms)	16	32	2,048	1,024
	17	16	2,048	1,024
	18	32	2,048	4,096
	20	64	4,096	1,024
	21	8	512	512
	22	16	1,024	1,024
Controls	43	8	32	64
(Ferrets maintained in isolation at the National Institute for Medical Research)	44	8	32	64
	45	16	32	32

* The sera were tested against Lee (B) virus with uniformly low results.
† Not tested.
N.B.—Pre-infection serum samples were not available.

tested against the standard Lee (B) virus but all showed a consistently low titre.

It will be seen that in both colonies the agglutinin antibody level to the current viruses was markedly raised. This appeared to be the direct result of infection and not the result of the non-specific inhibitory action often shown by normal ferret serum (Burnet and McCrea, 1946). In the case of the two attendants G and K the relatively high level of antibody to P.R.8. is probably due to lack of specificity in adult human serum as opposed to ferret serum. It seems likely that both these attendants were recently infected with influenza, as were the ferrets, and the evidence suggests that the current influenza A virus was the infective agent responsible.

The control sera were obtained from ferrets from the Farm Laboratories of the National Institute for Medical Research at Mill Hill. These animals had been kept in strict isolation and their antibody level was uniformly low. In the case of sera from animals of Colony B which showed no apparent symptoms the antibody level to the current strains was high, indicating a recent infection which had apparently been sub-clinical.

Two infected ferrets from colony A, were re-infected experimentally seven to eight weeks later, one with the standard A strain (P.R.8.) and the other with the 1947 A strain (BAR). In the former case (P.R.8.) the inoculation produced a sharp reaction with a temperature of 105·4° F. and some respiratory symptoms. The serum of this ferret tested with the P.R.8. and BAR antigens showed a pre-inoculation titre of 8 and 128 respectively and a convalescent titre of 1024 and 512. This indicated that the ferret was susceptible some eight weeks after the original infection and that the response to re-infection with P.R.8 was a rise in antibody to both P.R.8 and BAR.

In the case of the second ferret, re-infected with the BAR strain, there was no febrile reaction and no indication of infection; serological tests showed, however, that the pre-inoculation titre to P.R.8 and BAR was 8 and 128, and the convalescent titre 128 and 768 respectively. The slightly higher antibody level for the BAR strain in the pre-inoculation sample indicated a recent infection, and would also explain the higher degree of immunity to the homologous strain, *i.e.*, the current strain as opposed to P.R.8.

Ferrets which had recovered from the disease described above were susceptible to a virulent strain of canine distemper virus.

DISCUSSION

The indirect evidence suggests that the epizootic in the ferret colony was due to the current influenza virus A, the cause of influenza in the human population at the time. Previous work has demonstrated the susceptibility of ferrets to influenza, and it is probable that the source of the infection was the animal attendants who

were infected with the disease at this time. Once the infection had become established, spread was presumably direct from ferret to ferret, since the conditions under which the animals were maintained would be insufficient to prevent passage of the virus (Andrewes and Glover, 1941) ; moreover the attendants moving from court to court could readily convey infective nasal discharge with them.

The serological tests indicate that in both colonies the infection was very widespread and this is confirmed in Colony A by the large number of animals showing symptoms. In colony B since some of the animals showed high agglutinin titres but no symptoms, it is probable that these animals had a sub-clinical infection.

SUMMARY

The course of an epizootic of influenza in two ferret colonies is described. Haemagglutination tests of ferret convalescent sera would indicate that the strain of influenza A virus which occurred in the human population at this time was the causal organism.

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We are indebted to Mr. R. E. Glover, of the Farm Laboratories, National Institute for Medical Research, Mill Hill, N.W.7, for carrying out the immunity experiments against the influenza viruses and for many helpful suggestions in the preparation of this paper. We also wish to thank Mr. A. B. MacIntyre, M.R.C.V.S., of the Wellcome Physiological Research Laboratories.

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SOME OBSERVATIONS ON THE QUANTITATIVE STUDY OF VESICULAR STOMATITIS VIRUS

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INTRODUCTION

Vesicular stomatitis is a virus disease affecting horses, cattle and, more rarely, swine. The marked resemblance between the mouth lesions in particular of these two diseases suggested that a method of titration of suspensions of foot-and-mouth disease virus involving the development of lesions on the bovine tongue might be applicable to titrations of suspensions of vesicular stomatitis virus.

The quantitative study of the two viruses depends on the detection of the infective agent in progressive dilutions of the virus suspension by inoculation of a susceptible host. Owing to the strong species adaptation of strains of foot-and-mouth disease virus, the most sensitive test animal for a particular strain is one of the species for which that strain is adapted; thus, in titrations of strains of bovine origin and adaptation it is essential to use cattle for the test animals. In suggesting that this technique might be applicable to vesicular stomatitis virus it was necessary to determine, first, whether the actual method of inoculation and the reaction produced by the virus was suitable and, secondly, whether there was a sufficient degree of species adaptation of strains of vesicular stomatitis virus to necessitate the use of cattle for bovine strains or whether another species of test animal of more convenient size and cost would give as accurate results.

METHODS

The use of cattle for titration of suspensions of foot-and-mouth disease virus has been fully described elsewhere (Henderson, 1945) and reference will be made only to those points necessary for explanation in an attempt to apply the technique to vesicular stomatitis virus. Briefly, the routine method consists of the simultaneous intradermal inoculation of the tongue of two cattle with four dilutions using five inoculation sites on each tongue for each dilution, thus providing ten observations for each of four dilutions. The 50 per cent. positive end-point is then calculated by the method of Reed and Muench (1938).

Strains of vesicular stomatitis virus. There is no record of the isolation in Great Britain of the virus of vesicular stomatitis and the strains used in this work were obtained from the United States of

America through the courtesy of Dr. H. W. Schoening of the U.S. Department of Agriculture's Bureau of Animal Industry.

Strain Ind.-C (American identification). Indiana type. Isolated in 1942 from an outbreak in Colorado. Bovine tongue epithelium received at Pirbright in December, 1944, and since passaged in cattle.

Strain N.J.-M (American identification). New Jersey type. Isolated in 1943 from an outbreak in a swine serum production establishment in Kansas City (see Schoening and Crawford, 1945). The subsequent passaging of this strain has been rather varied and although bovine tongue epithelium was received in December, 1944, the earlier history of the strain included some passages in guinea-pigs (see Shahan, Frank and Mott, 1946). The strain has since been passaged in cattle at Pirbright.

Preparation of virus suspensions and dilutions. The infective material used was vesicle epithelium collected from unruptured primary lesions of cattle inoculated intradermally on the tongue. This epithelium was minced, ground in a mortar with the addition of sand and 0·9 per cent. sodium chloride solution to make a 1 : 10 saline suspension which was then centrifuged for 10 minutes at 2,000 r.p.m. The supernatant was used for the preparation of dilutions in 0·9 per cent. sodium chloride solution and was taken as the starting-point for the dilution series.

Test animals and methods of inoculation

Cattle. Devon steers, one and a half to two years old, were used following narcotisation by the i/v injection of 40 c.c. of a 7·5 per cent. solution of Pentothal (Abbott brand of thiopentonum solubile B.P.). Four dilutions of the virus suspension were inoculated intradermally on the tongue, five sites for each dilution. They were arranged systematically with the lowest nearest the dorsal prominence and the highest nearest the tip of the tongue (Fig. 1).

Horses. Exmoor ponies, two to four years old, were anaesthetised by the i/v injection of 20 c.c. of a 10 per cent. solution of Pentothal and inoculated in exactly the same manner as the cattle except that on the narrower tongue of the pony it was only possible to inoculate four sites with each dilution.

Swine. Middle White store pigs of about 30 kg. weight were narcotised by the intraperitoneal injection of 10 c.c. of a 20 per cent. solution of Pentothal and inoculated by scarification of the snout and intradermal inoculation of the tongue. Only one dilution was used for each animal and no attempt was made to obtain more than one observation.

Guinea-pigs of the Institute stock (450 to 500 g.) were inoculated intradermally on the large hairless pad of both hind feet. Groups of eight were used for each dilution, one observation being obtained from each guinea-pig.

In the above animals the development of a lesion at a site of inoculation constituted a positive observation.

Fertile hen eggs incubated for seven days at 39° C. were inoculated by the chorioallantoic membrane route and thereafter incubated at 35° C. This age of embryo and temperature of incubation after inoculation was reported by Sigurdsson (1943) to be the most suitable for growth of vesicular stomatitis virus. Batches of four or five eggs were used for each dilution. In other experiments in which these virus strains have been passaged in eggs, abnormality of the chorioallantoic membrane has never been observed ; death of the embryo occurred regularly, however, in one or two days and this lethal effect was the criterion of infection in the present experiments, any doubt about the cause of death being checked by further passage to eggs and guinea-pigs.

Mice. One titration using mice was done by my colleague, Mr. H. H. Skinner. White mice, three to four weeks old, were inoculated with 0·03 c.c. intracerebrally during A.C.E. mixture anaesthesia. Groups of six mice were used for each dilution. Inoculation of infective material produced paralysis and death in two to five days.

RESULTS

Three titrations of vesicular stomatitis virus have been performed in cattle using the routine method already described. In each case the reaction of the cattle to the simultaneous inoculation of four dilutions of virus was sufficiently like the reaction to foot-and-mouth disease virus to enable the result of the titration to be determined. In some cases the individual lesions at the inoculation sites were less easy to read than those of foot-and-mouth disease as in their early stages there was less vesiculation, the site of reaction being merely an area of blanched epithelium ; other sites of reaction, however, consisted of quite well-developed vesicles. The rate of development of the lesions was, in general, slower than with foot-and-mouth disease and, as had been noted in the reaction of the cattle used for passaging the strains, there was little tendency for the development of secondary lesions. With foot-and-mouth disease in cattle the development of secondary lesions within 12 to 24 hours of the appearance of the primary lesions of a titration necessitates the completion of observations before this complication occurs whereas with these titrations of vesicular stomatitis virus the absence of generalisation enabled observations to be continued into the second day after inoculation. The following example gives the result of a titration in detail. (Fig. 1.)

Table I shows the percentage of positive observations for each dilution from which it can be calculated that the 50 per cent. positive end-point is $10^{-3\cdot6}$.

Having determined that the foot-and-mouth disease titration method in cattle could be successfully used for the quantitative study of vesicular stomatitis virus it was necessary to do comparative titrations using cattle and other hosts. Two such experiments have

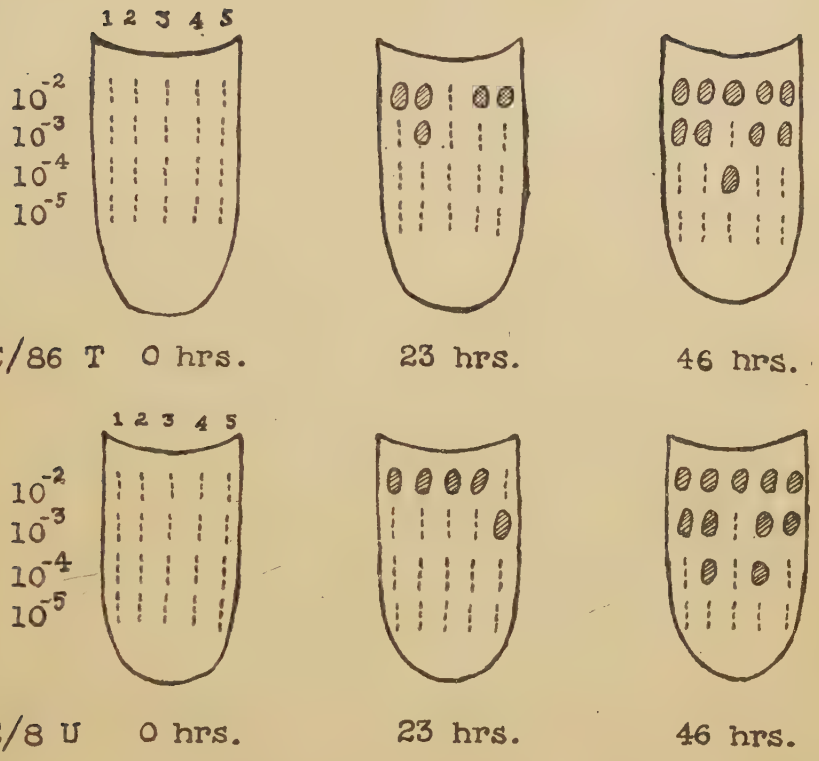

FIG. 1

Simultaneous multiple inoculation of four dilutions of a tenfold series, five sites per dilution. The dotted lines indicate the sites of inoculation, the shaded areas show the sites of reaction.

Titration of a 1/10 centrifuged suspension of 4th Pirbright bovine passage strain Ind.-C. epithelium.

Steer No. C/86 T, Fig. 1.

Time hours	*Temperature ° F.*	*Remarks*
0	—	Inoculated i/d 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions, five sites per dilution
23	101	10^{-2}, reactions at sites 1, 2, 4 and 5 10^{-3}, reaction at site 2
46	102	10^{-2}, reactions at sites 1, 2, 3, 4 and 5 10^{-3}, reactions at sites 1, 2, 4 and 5 10^{-4}, reaction at site 3 10^{-5}, negative
120	—	No secondary lesions observed

Steer No. C/8 U, Fig. 1.

Time hours	*Temperature ° F.*	*Remarks*
0	—	Inoculated i/d 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions, five sites per dilution
23	104	10^{-2}, reactions at sites 1, 2, 3 and 4 10^{-3}, reaction at site 5
46	101	10^{-2}, reactions at sites 1, 2, 3, 4 and 5 10^{-3}, reactions at sites 1, 2, 4 and 5 10^{-4}, reactions at sites 2 and 4 10^{-5}, negative
120	—	No secondary lesions observed

TABLE I

RESULT OF TITRATION ILLUSTRATED IN FIG. 1

Dilutions	*Observations*		*Accumulations*		*Per cent. positive*
	Positive	*Negative*	*Positive*	*Negative*	
10^{-2}	10	0	21	0	100
10^{-3}	8	2	11	2	85
10^{-4}	3	7	3	9	25
10^{-5}	0	10	0	19	0

been done, one with strain N.J.-M and the other with strain Ind.-C. The results are summarised in Tables II and III respectively.

The following are the most important points of the results of these two experiments. The close agreement of the end-points obtained from cattle and from guinea-pigs is in marked distinction

TABLE II

VESICULAR STOMATITIS VIRUS. TITRATION OF A 1/10 SUSPENSION OF 4TH PIRBRIGHT BOVINE PASSAGE STRAIN N.J.-M. EPITHELIUM IN CATTLE, SWINE, GUINEA-PIGS AND FERTILE HEN EGGS

Dilutions	*Observations*							
	Cattle		*Swine*		*Guinea-pigs*		*Hen eggs*	
	+	—	+	—	+	—	+	—
10^{-1}					8	0		
10^{-2}	10	0	1	0	8	0	2	3
10^{-3}	7	3	0	1	6	2	3	1
10^{-4}	1	9	0	1	1	7	0	4
10^{-5}	0	10	0	1	0	8	0	4
10^{-6}							0	4
50 per cent. positive end-point	$10^{-3.4}$		Insufficient observations		$10^{-3.4}$		$10^{-2.7}$	

TABLE III

VESICULAR STOMATITIS VIRUS. TITRATION OF A 1/10 SUSPENSION OF 8TH PIRBRIGHT BOVINE PASSAGE STRAIN IND.-C. EPITHELIUM IN CATTLE, HORSES, GUINEA-PIGS, MICE AND FERTILE HEN EGGS

Dilutions	*Observations*									
	Cattle		*Horses*		*Guinea-pigs*		*Mice*		*Hen eggs*	
	+	—	+	—	+	—	+	—	+	—
10^{-2}	10	0	8	0	7	1*	6	0	4	0
10^{-3}	9	1	8	0	8	0	6	0	5	0
10^{-4}	6	4	1	7	8	0	6	0	1	4
10^{-5}	4	6	1	7	2	6	0	6	0	5
50 per cent. positive end-point	$10^{-4.4}$		$10^{-3.6}$		$10^{-4.6}$		$10^{-4.5}$		$10^{-3.6}$	

* Dead on the 4th day after inoculation.

to the results of similar titrations of strains of foot-and-mouth disease virus of bovine adaptation in which we have found that the end-point in guinea-pigs is invariably ten to a thousandfold lower than in cattle. The low end-points obtained from eggs is of interest as, once the virus has been passaged serially by the chorioallantoic membrane route, the chick embryo appears to be very sensitive to the virus. The method of inoculating cattle is just as applicable to inoculation of the horse. The reaction of the mice to intracerebral inoculation suggests that by this route this host may be as sensitive for detection of the virus as cattle, horses and guinea-pigs inoculated intradermally.

DISCUSSION

In spite of the small amount of time and material that it has been possible to devote to this work some useful conclusions can be drawn. The similar susceptibility of cattle and guinea-pigs to the two strains used makes it apparent that it is not necessarily important to use cattle for titration of strains of bovine adaptation. It is perhaps unnecessary to stress that the species adaptation of virus strains is a variable characteristic and what is true for the two strains examined will not necessarily apply to all strains. If, however, a strain of vesicular stomatitis virus having a strong species adaptation for horses or cattle has to be studied quantitatively, then there is available a method of titration using either horses or cattle. Little comment can be made on the result of the inoculation of the four swine with dilutions of strain N.J.-M. These animals were available at the time of the test and as this strain had been originally isolated from swine, they were included as they might have demonstrated some particular adaptation of the strain to this host. As, however, the non-reactors all received dilutions that failed to produce completely positive results in other species, no conclusion can be drawn.

In both the experiments shown in Tables II and III the egg was less satisfactory for the detection of virus than cattle or guinea-pigs, the end-points obtained were lower and any suggestion of a non-specific death of an embryo had to be checked by further inoculations. When the virus is given a number of serial passages in eggs we find that the titre of egg material in eggs is very high, say 10^8. Burnet and Galloway (1934), working with guinea-pig and egg passaged strains, found eggs more sensitive than guinea-pigs for detection of minimal infective doses, but the relative susceptibility of other species when used for titration of egg passaged material has yet to be studied in detail.

The data provided by these experiments are insufficient for discussion of the error of any of these methods of titration of vesicular stomatitis virus, but this aspect of quantitative studies in which cattle are used is discussed at length in connection with the titration of foot-and-mouth disease virus (Henderson, 1945) as well as

particular points such as the choice of a systematic arrangement of the dilution sites on the bovine tongue instead of randomisation, the susceptibility of different areas of the tongue and reports of attempts at improvements in technique.

SUMMARY

The method evolved for the titration of foot-and-mouth disease virus in cattle is suitable for vesicular stomatitis virus in either cattle or horses.

In titration of foot-and-mouth disease virus of bovine adaptation, it is essential to use cattle to obtain an accurate result, but this is not necessarily so for vesicular stomatitis virus as guinea-pigs were as sensitive as cattle for detection of the virus in serial dilutions of the two strains examined.

Although following serial passage of vesicular stomatitis virus in the fertile hen egg a very high titre can be obtained in eggs, it was found that for titration of bovine material of the two strains examined the egg was not so satisfactory as cattle or guinea-pigs.

ACKNOWLEDGMENT

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THE TYPING OF STAPHYLOCOCCI OF ANIMAL ORIGIN BY THE BACTERIOPHAGE METHOD

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INTRODUCTION

Wilson and Atkinson (1945) devised a method whereby staphylococci could be classified into a number of different types by their sensitivity to bacteriophage. This method has proved to be of considerable value in the investigation of outbreaks of staphylococcal infection in human subjects. Williams Smith (1948a, b) showed that acquired phage resistance was responsible for many strains being classified as different phage types. As a result of this finding, he attempted to classify staphylococci, by phage methods, into types that differed other than by virtue of acquired phage resistance.

It was decided to ascertain whether the method of Wilson and Atkinson might be of value in the epidemiological study of staphylococcal infection in animals. The two most suitable diseases for study were thought to be bovine mastitis and pyaemia in lambs. When this work was nearing completion, Macdonald (1946), using phages provided by Wilson and Atkinson, reported the typing of 150 strains of *Staph. aureus* obtained from bulk samples of accredited milk and 34 strains from cases of bovine mastitis. Of the strains from bulk milk samples, 123 were fully susceptible to phage 42D and 12 to other phages; 15 were untypeable. The 34 mastitis strains belonged to phage 42D type.

TECHNIQUE

The technique of bacteriophage typing was that described by Wilson and Atkinson (1945) with a few slight modifications (Williams Smith, 1948a).

The following 21 phages received from Professor G. S. Wilson were used for typing: 3A, 3B, 3C, 6, 7, 29, 29A, 31, 42B, 42C, 42D, 42E, 44, 44A, 47, 47A, 47B, 47C, 51, 52 and 52A. Eleven others, prepared during the course of this work, were also used. These were phages E174/16, 14/94, 1363/14, 129/16, 30/16. P42D/E193, S7, G12, X2, 88 and 88A. Some were grouped together because strains that could be distinguished from each other only by their reactions with phages of the *same* group were believed to owe their difference in behaviour to acquired phage resistance (Williams Smith, 1948a, b). These groups were:—

3A Group: Phages 3A, 3B, 3C and 51.
6 Group: Phages 6, 7, 42B, 47 and 47C.
29 Group: Phages 29, 29A, 31, 42C, 44, 52 and 52A.

42D Group : Phages 42D, E174/16, 14/94, 1363/14, 129/16, 30/16 and P42D/E193.

88 Group : Phages 88 and 88A.

The scheme of notation used when reading tests was as follows : Confluent lysis with no secondary growth, CL ; confluent lysis with secondary growth, +++ ; numerous semi-confluent plaques, ++ ; discrete plaques, + ; less than 20 plaques, ±.

RESULTS

The phage-typing of staphylococci isolated from bovine milk samples. —The total number of strains of coagulase-positive staphylococci that was examined from milk samples of individual cows was 1,016, of which 948 (93·3 per cent.) were phage-typed and 68 (6·7 per cent.) proved to be susceptible to all the test phages. Of these 1,016 strains, 852 (83·8 per cent.) were insusceptible to one or more of the phages of the 42D group, and 96 (9·5 per cent.) to one or more of the 29 group. Two strains were susceptible to phage X2, one to phage 42B, and one to phages 3A, 3B and 3C.

Table I shows, in detail, the results of phage-typing 200 strains of coagulase-positive staphylococci. These strains, unlike the 1,016 referred to above, were selected so as to include no more than one strain from any one herd of cows. Consequently, this table illustrates more accurately the frequency distribution of the phage types. Of these 200 strains, 140 (70·0 per cent.) were susceptible to one or more of the 42D group of phages, 42 (21·0 per cent.) to one or more of the 29 group ; one to phage X2, one to phage 42B, and one to phages 3A, 3B, 3C ; 15 strains (7·5 per cent.) were untypeable. Apart from the 46 strains (23·0 per cent.) that were susceptible to *all* the phages of the 42D group, none of the 25 phage types comprised more than 9 per cent. of the whole. Of the 140 strains of the 42D group, 47 were not fully susceptible to Wilson and Atkinson's phage 42D, and if the other six phages of this group, which were prepared during the course of this work, had not been used in routine typing, these 47 strains, comprising 23·5 per cent. of the whole, would have been regarded as untypeable.

Seventy-six of the 200 strains referred to above were isolated from the milk of cows suffering from acute or chronic mastitis. Phage-typing did not serve to distinguish these strains from those that were isolated from the milk of cows with apparently normal udders.

Phage-types of staphylococci in milk samples of cows in the same herd.—The strains, obtained from 24 herds, comprised the majority of the 1,016 mentioned above. Series were isolated from milk samples of cows in the same herd on one or several occasions. Only one strain was examined from any one milk sample, the majority of which were quarter samples. It was usual to find that one phage type of staphylococcus was predominant in the strains examined from each herd, but was different in individual herds. In each of

Table I

Typing of 200 Strains of Staphylococci Isolated from Bovine Milk Samples

Phage type	*Phage filtrates in test dilution*																*Strains of staphylococci*	
	A	B	C		A			A	D	14	1363	129	E174	30	P42D	B			
	3	3	3	29	29	31	52	52	42	94	14	16	16	16	E193	42	X2	*No.*	%
1	CL	CL	CL																
2				—	CL	CL	CL	CL										1	0·5
3				—	+	CL	CL	CL										7	3·5
4				—	CL	—	CL	++										2	1·0
5				—	—	—	CL	CL										6	3·0
6				—	—	—	CL	+										5	2·5
7				+	CL	—	+	+										4	2·0
8				CL	—	—	—	—										9	4·5
9				—	+	CL	+	+										1	0·5
10				—	CL	CL	—	—										6	3·0
11																		2	1·0
12									CL	CL	CL	CL	CL	CL	CL			46	23·0
13									CL	CL	+	—	CL	—	CL			18	9·0
14									—	+	—	—	CL	—	CL			5	2·5
15									CL	CL	+	CL	CL	—	CL			9	4·5
16									—	CL	CL	—	CL	CL	CL			6	3·0
17									—	CL	—	—	CL	—	CL			10	5·0
18									CL	—	—	CL	—	—	CL			6	3·0
19									CL	—	—	—	—	—	—			1	0·5
20									CL	—	CL	CL	—	+	+			6	3·0
21									CL	CL	CL	CL	CL	—	CL			7	3·5
22									—	+	—	—	—	—	CL			7	3·5
23									+	—	—	CL	—	—	CL			6	3·0
24									—	CL	CL	—	CL	—	CL			13	6·5
25																CL		1	0·5
																	CL	1	0·5
											Untypeable							15	7·5

The degree of lytic action is designated as indicated in the text.
The phage types have been numbered only for the purpose of this table and are not recognised designations.

23 herds the predominant phage type present was one of the phage types of the 42D group. In the remaining herd, from which 15 strains were examined, ten were susceptible to phages 29A and 31, and five to all of the phages of the 42D group.

It was not unusual to find two quarters of the same cow infected with different phage types of staphylococci, but strains that had been isolated from the same quarter of the same cow on different occasions were usually of the same phage type although occasionally different types were found. It was thought that the reason for this was that either the quarter had become infected with another phage type of staphylococcus during the interval between two successive examinations of the milk, or, as only one strain was submitted to phage-typing from each milk sample, two phage types had been present in the same quarter at the same time.

The presence of more than one phage type of staphylococcus in the same quarter.—Twenty-six milk samples from different quarters of different cows known to be harbouring staphylococci were plated on blood agar. Six coagulase-positive colonies from each plate were picked off into nutrient broth, and phage-typed in the usual manner. The six strains from each of 22 (84·6 per cent.) of the milk samples were of the same phage type. Four (15·4 per cent.) were shown to contain two different phage types ; only one contained two phage types of the 42D group, while three contained a phage type of the 42D group with either a phage type of the 29 group (two samples) or an untypeable strain (one sample).

Further studies on three strains of the 42D group.—Further studies were made on three strains of the 42D group as they were of special interest. Strain 1363, the propagating strain of phage 42D, had been incriminated in an outbreak of food poisoning following the eating of ice-cream (Williams *et al.*, 1946), strain 823 had been isolated from a case of human mastitis and strain 16 from a case of bovine mastitis. The lytic action of the 42D group of phages on these strains is shown below :—

	Test phages						
	42D	E174	14	1363	129	30	P42D
		16	94	14	16	16	E193
Strain 16 . .	CL	CL	CL	CL	CL	CL	CL
,, 1363 . .	CL	—	—	—	—	—	—
,, 823 . .	CL	—	—	—	—	—	—

Strain 1363 was lysogenic and strain 16 was non-lysogenic. Strain 823 was the only non-lysogenic strain that had been encountered which was susceptible to one but not to all the phages of the 42D group. This fact together with the reactions of the three

strains with the test phages made it possible to distinguish the three strains from each other. Colonies on sheep blood agar were surrounded by a wide, semi-clear zone of β haemolysis, a property usually associated with staphylococci of animal origin.

Antigenic analysis.—The three strains were shown by cross-absorption tests to be antigenically identical. The agglutination tests were carried out in tubes as well as by the slide technique of Cowan (1939).

Artificial infection of the bovine udder.—A Shorthorn cow, one month after the commencement of her first lactation, was used for this experiment. Milk from each quarter was examined several times over a period of 14 days and no staphylococci or other bacteria associated with mastitis were found. The four quarters were milked out completely and the following materials injected into them.

Left fore-quarter ... 1 ml. of an 18-hour broth culture of strain 1363.
Left hind-quarter ... 1 ml. of an 18-hour broth culture of strain 823
Right hind-quarter ... 1 ml. of an 18-hour broth culture of strain 16.
Right fore-quarter ... 1 ml. of sterile broth.

The injections were made with full sterile precautions using a 1 ml. syringe without a needle, the nozzle being inserted through the teat sphincter. The cow was not milked for eight hours, after which she was milked twice daily, the operator using a sterile rubber glove for each quarter. Apart from pyrexia, some slight swelling of the udder and the presence of clots in the milk from the three infected quarters, no other signs of infection were noted. These signs subsided completely within 48 hours, but staphylococci were isolated from the left fore-quarter, the left hind-quarter, and the right hind quarter at each bi-weekly test for the next six weeks, when the experiment was terminated. Staphylococci were never isolated from the right fore-quarter. Three strains obtained from each quarter at each test were phage-typed ; no evidence of cross-infection from one quarter to another was noted. Two plate counts were made on the milk obtained from each quarter at 60 hours and 26 days after infection. The results are shown below.

NUMBER OF STAPHYLOCOCCAL COLONIES (APPROX.) PER ML. OF OF MILK

Quarter	*Left fore*	*Left hind*	*Right hind*	*Right fore*
60 hours	160,000	240,000	100,000	0
26 days	20,000	19,000	13,000	0

The phage-typing of strains of ovine origin.—Two hundred and ninety-seven strains of ovine origin were isolated from cases of lamb pyaemia, mastitis in ewes and other types of staphylococcal infection, and from swabs taken from the skin and other sites of sheep. The results showed that 209 (70·4 per cent.) were typeable, 62 (20·9 per

cent.) were partially susceptible to some of the test phages, and 26 (8·7 per cent.) were insusceptible to all the test phages (Table II).

Lamb pyaemia investigations.—Sixty-six (76·8 per cent.) of the 86 strains isolated from cases of lamb pyaemia were typed; 15 (17·5 per cent.) were partially susceptible to some of the test phages and five (5·7 per cent.) were insusceptible to all the test phages. The results are illustrated in Table III.

TABLE II

THE PHAGE-TYPING OF 297 STRAINS OF STAPHYLOCOCCI OF OVINE ORIGIN

	Phage filtrates in test dilution					*Strains of staphylococci*	
Phage type	X2	S7	88	88A	42C	*Number*	%
1	CL					105	35·4
2		CL				33	11·1
3			CL			38	12·8
4			CL	CL		15	5·1
5				CL		17	5·7
6					CL	1	0·3
			Untypeable			88	29·6

The phage types have been numbered only for the purpose of this table and are not recognised designations.

TABLE III

THE PHAGE-TYPING OF 86 STRAINS OF STAPHYLOCOCCI ISOLATED FROM CASES OF LAMB PYAEMIA

	Phage filtrates in test dilution					*Strains of staphylococci*	
Phage type	X2	S7	88	88A	42C	*Number*	%
1	CL					33	38·4
2		CL				12	13·9
3			CL			7	8·2
4			CL	CL		7	8·2
5				CL		6	6·9
6					CL	1	1·2
			Untypeable			20	23·2

The phage types have been numbered only for the purpose of this table and are not recognised designations.

A predominant phage type was not usually found among strains of staphylococci isolated from different cases of lamb pyaemia occurring in the same flock. More often several types were present. For example, the strains isolated from seven cases in one flock belonged to the following types; X2 (three strains), S7 (one strain), 88 (one strain), 88-88A (one strain), untypeable (one strain): from five cases in another flock; S7 (two strains), X2 (one strain), 88 (one strain), 88A (one strain). In two cases, the strains isolated from different lesions in the same carcase belonged to different

phage types. A small number of strains isolated from skin swabs of affected animals were examined ; the same phage type was found on the skin as in the actual lesions.

A total of 175 strains of staphylococci was received from Mr. A. Foggie, of the Moredun Institute, Gilmerton, who reported that 140 had been isolated periodically from the skin, nose, mouth, and vagina of ewes in two flocks during a period comprising, roughly, the first five months of the year 1944, and 30 from the skin of their lambs (Foggie, 1947). Each flock consisted of 20 ewes. Flock A, in which lamb pyaemia was known to occur, was tick infested ; flock B, in which it had never been observed, was free from infestation. The carrier rate of staphylococci was approximately equal in both flocks. No typical cases of lamb pyaemia developed in flock A, but staphylococcal skin abscesses were observed in two lambs and three ewes. The majority of the strains obtained from both flocks belonged to the following phage types : X2, S7, 88, 88A, 88/88A. Different phage types of staphylococci were often found in different sites in the same sheep and in the same site on different occasions. Of the five strains isolated from the abscesses in the two lambs and three ewes of flock A, two were of phage X2 type, two of phage 88 type and one of phage S7 type. A staphylococcal strain isolated from a pustule on a finger of a worker concerned with these field investigations of lamb pyaemia was of phage 88A type.

DISCUSSION

Macdonald (1946) found that 123 of the 150 strains he examined from bulk milk were of one phage type, 42D. However, the use of seven phages in this present work instead of only phage 42D, has resulted in strains of this type and others previously regarded as untypeable being satisfactorily differentiated into 13 phage types. The use of these phages with those received from Professor G. S. Wilson has enabled the strains of staphylococci that have been examined from bovine milk samples to be classified into 25 phage types. Of the 1,016 strains that were isolated from bovine milk samples, all except four of the typeable strains were susceptible to one or the other of two groups of phages, 83·8 per cent. being susceptible to one or more of the 42D group of phages and 9·5 per cent. to one or more of the 29 group. In the case of the 200 strains that were epidemiologically unrelated, these percentages were 70 and 21, suggesting that strains susceptible to one or more of the 29 group of phages occurred only sporadically in herds. This was borne out by the results of the examination of strains isolated from milk samples of cows in the same herd ; strains of the 29 group were present in the milk of a few of the cows in nearly all the herds but only in one herd did they constitute the predominant type.

Strains susceptible to phage 42D and one or more of the 29 group of phages have also been found by Wilson and Atkinson (1945, personal communication), Allison (personal communication),

and others in human subjects. Phage 42D type strains were, however, not found in milk so frequently as they were by Macdonald (1946) and in the present work. The fact that three 42D type strains, one isolated from an outbreak of staphylococcal food poisoning in man, one from a case of human mastitis and one from bovine mastitis, were antigenically identical and were able to maintain themselves in the bovine udder for six weeks is additional evidence supporting the view that some strains may be capable of infecting man as well as cows and that milk may be a source of human staphylococcal infection.

The discovery of two phage types in each of four out of 26 milk samples obtained from different quarters of different cows indicated that, in epidemiological studies of bovine mastitis, several staphylococcal cultures from each milk sample would have to be submitted to phage-typing. This would be a laborious task. It is even possible that, if more than six cultures had been tested from each milk sample, a higher percentage would have been found to be infected with more than one phage type.

Earlier work (Williams Smith, 1948b) has shown that when two strains of the 42D group, one of which was lysogenic to the other, were grown together in broth the susceptible strain underwent a change in phage type. Evidence was adduced to suggest that this change might take place in the field. The majority of strains of the 42D group were also shown to be carrying phages that were lytic to other strains of this group. (Williams Smith, 1948a). As over 80 per cent. of the 1,016 strains that were examined were susceptible to one or more of the phages of the 42D group, it is possible that change of type may be a complicating factor in the epidemiological study of bovine mastitis.

Unlike the bovine strains, only one of the 297 staphylococcal strains of ovine origin was susceptible to the phages of Wilson and Atkinson and new phages had to be prepared to identify them. Only two bovine strains were susceptible to these new phages and Allison (personal communication), who has used two of them, X2 and S7, in the routine typing of staphylococcal strains of human origin, has not encountered any strains that are fully susceptible. One strain isolated from a pustule in the skin of a worker concerned with field investigations on lamb pyaemia was, however, found to be susceptible to one of these new phages (88A). Thus, with a few exceptions, staphylococci of ovine origin, at least from the point of view of phage susceptibility, are distinct from those of human or bovine origin.

Although about 50 per cent. of the ovine strains that were typeable were of phage X2 type, many of these strains and some of the untypeable strains were lysogenic to the propagating strain of phage X2. It is probable, therefore, that they could be sub-divided in a similar manner to the 42D type strains (Williams Smith, 1948a), *i.e.*, by propagating the phages carried by some of the lysogenic strains and using them in routine tests.

Several phage types of staphylococci were often isolated from different lamb pyaemia cases occurring in the same flock and were also present on the skin of normal sheep and lambs. The same phage type was also found on the skin and in the actual lesions in the same lamb. These findings do not conflict with the view that lamb pyaemia is an inoculation infection, the staphylococci present on the skin of the lambs being inoculated into the tissues of the lamb by the bite of a tick.

SUMMARY

Investigations have been carried out to determine whether the bacteriophage method of typing staphylococci developed by Wilson and Atkinson (1945) could be applied to the epidemiological study of staphylococcal infections in animals.

Of 1,016 strains isolated from bovine milk samples, 93·3 per cent. were phage-typed and 6·7 per cent. were untypeable. Of 200 strains that were epidemiologically unrelated, 92·5 per cent. could be classified into 25 different phage types. One of these types comprised 23·0 per cent. of the 200 strains examined, but none of the other phage types comprised more than 9·0 per cent. of the whole. It was usual to find one type predominant in the strains isolated from the milk samples of any one herd. Phage-typing did not distinguish strains isolated from cows with mastitis from those found in milk samples from apparently normal udders. Milk samples from each of four out of 26 quarters known to be infected with staphylococci contained two phage types. It is considered that phage-typing may have its limitations in the epidemiological study of bovine mastitis.

Three strains of staphylococci, one incriminated in an outbreak of food poisoning in man, one from a case of human mastitis and one from a case of bovine mastitis, were susceptible to the same phage (42D), were antigenically identical and were able to maintain themselves in the bovine udder for a period of six weeks.

Unlike the bovine strains, all except one of the 297 strains of ovine origin that were examined were insusceptible to the phages of Wilson and Atkinson. By the use of new phages, 70·4 per cent. were typed, 20·9 per cent. were partially susceptible to some of the phages and 8·7 per cent. were insusceptible to all of them. Six phage types were identified, one of which comprised 35·4 per cent. of the 297 strains. Observations made on lamb pyaemia did not conflict with the view that it is an inoculation infection, the staphylococci present on the skin of the lambs being inoculated into the tissues of the lamb by the bite of a tick.

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THE OCCURRENCE OF NEWCASTLE DISEASE IN EGYPT

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INTRODUCTION

According to the literature, classical fowl-plague has been enzootic in Egypt for a great many years, the large annual recruitment of the flocks from native incubators and hatcheries apparently providing sufficient susceptible birds to support the development of major epizootics in most years.

From time to time workers have remarked on differences in the symptomatology of certain outbreaks, usually in the direction of a lengthening of the incubation period and course of the disease. Such differences prompted Lagrange (1929) to describe what he termed " Egyptian pseudo-fowl-plague." Purchase (1931) showed by means of cross-immunity tests that Lagrange's virus was identical immunologically with strains of classical fowl-plague, and that in symptomatology, pathology, and pathogenicity of the virus for other species the disease might be regarded as a form of fowl-plague that runs a slower course and in which the blood contains a lower concentration than usual of virus. These characters were maintained by the virus through a number of experimental passages. From a review of incubation periods, course, and symptomatology of strains isolated at the Giza Laboratory over a number of years, Carpano (1933) reached the same conclusion.

Rashad (1934), however, obtained a strain of Newcastle virus from Doyle for the purpose of making comparative tests. According to his account, this sample killed two fowls in nine days, while four fowls subinoculated with the blood of the original two also died in seven to nine days. The spleens of these four birds were used to prepare vaccine by Staub's method and eight hens were vaccinated. The eight birds were then tested with blood containing the virus of fowl-plague and all survived. The serum of geese immunised against Egyptian fowl-plague is said to have neutralised Doyle's virus, *i.e.*, the virus of Newcastle disease, and conversely the serum of geese immunised with Newcastle disease virus neutralised the virus of fowl-plague (no numbers, details or protocols given). Rashad concluded " The virus of Newcastle disease may be a weak strain of fowl-plague that was once virulent to pigeons and has gradually lost this property." Rashad remarks that none of the birds inoculated with Doyle's virus showed the respiratory symptoms and lesions characteristic of Newcastle disease, but in another part of the paper he states " in the present investigation of the different strains, we came across cases resembling Newcastle disease." It is impossible to place any satisfactory interpretation on Rashad's findings.

Certain observers have expressed the opinion that during the last few years a new train of respiratory symptoms and lesions has appeared in some

of the outbreaks, and that vaccination with tissue vaccines, prepared according to the methods of Todd and others, has frequently been quite unsuccessful in protecting the birds. The possibility that the disease might be infectious laryngo-tracheitis has been canvassed as usual. Finally, it may be of interest to record that early in this investigation, we were informed by one poultry dealer that a few years ago a new disease characterised by respiratory symptoms made its appearance. The dealers call it " Shohhaka " or " Zoggotta," which means gasping or hiccoughing, in reference no doubt to the characteristic gasping cry of the sick birds.

CURRENT OBSERVATIONS

In 1946 three strains of " fowl-plague " virus, which will be referred to as A, C, and G, were being maintained by Dr. Bishlawy for tissue-vaccine production at the Serum Institute. By purchasing from dealers, it was his practice to assemble a number of healthy birds—often an exceedingly difficult matter because of the widespread incidence of infection. Batches of 20 to 50 birds were then inoculated with glycerin-preserved material of one of the stock-strains of virus, and organs for tissue-vaccine production were collected from freshly dead birds, or birds killed *in extremis*. There were no adequate facilities for isolation, and not infrequently—under the pressure of demands for vaccine—inoculations with two strains of virus might overlap, or inoculations might be in progress at the same time as natural cases of the disease were occurring in other fowls recently purchased from dealers. Under such conditions the risks of accidental cross-infection between strains were considerable.

In the present investigation a supply of the Muktesar vaccine-strain of Newcastle virus was obtained from Palestine, through the courtesy of Mr. H. R. Binns. Fifteen healthy fowls were inoculated with the normal dose of 1 c.c. of a 10^{-3} dilution of allantoic fluid, and the vaccinated birds were challenged at intervals varying from five to 14 days with one of the three stock strains (Table I). Since it appeared to have the characters of a Newcastle virus, the " C " stock strain was the first to be employed, and it will be observed that the vaccine afforded complete protection against this strain. Against the " A " strain, which was collected in 1945 and which is regarded as the most virulent strain of classical fowl-plague so far obtained, no protection could be demonstrated; but with the " G " strain the results were difficult to assess, since the three vaccinated birds survived ten to eleven days after the challenge inoculation, whereas the single control died in 48 hours. The absence of proper facilities for isolation has already been remarked, and it may well be that the late deaths of the " G " birds were caused by accidental infection with the " A " virus, since the six birds originally tested with " C " virus had, unfortunately, been reinoculated with " A " virus on the same day as the " G " virus tests were made, and deaths were occurring among these birds and among the controls from the fifth day onwards.

TABLE I

CHALLENGE OF NEWCASTLE-VACCINATED BIRDS WITH STOCK STRAINS OF FOWL-PLAGUE VIRUS

Number of birds	*Vaccination*	*Interval in days*	*Challenge*	*Results*
6	Newcastle vaccine, 1 c.c. 10^{-3}	5	"C" virus, 0·001 c.c. tissue suspension	All survived.
2	Controls	—	"	*D5, D5.
6	Newcastle vaccine, 1 c.c. 10^{-3}	9	"A" virus, 0·001 c.c. tissue suspension	D3, D4, D4, D5, D5, D5.
2	Controls	—	"	D4, D5.
3	Newcastle vaccine, 1 c.c. 10^{-3}	14	"G" virus 0·001 c.c. tissue suspension	D10, D10, D11
1	Controls	—	"	D2
†6	Newcastle vaccine, 1 c.c. 10^{-3}	14	"A" virus, 0·001 c.c. tissue suspension	D5, D7, D8, D9, D10, D11.
5	Controls	—	"	D5, D5, D5, D6, D13.

* D5 signifies one death on the fifth day after the challenge inoculation. This system is followed in all the tables.

† Previously challenged with "C" virus on the fifth day after vaccination, shown at top of table.

It seemed likely then that the "C" strain was a Newcastle disease virus and the "A" strain a classical fowl-plague virus. The test with the "G" strain was regarded as inconclusive. Further tests with "A" virus confirmed that the Newcastle disease vaccine did not protect against this strain.

It was then decided (for reasons that will be obvious) not to introduce a fully virulent Newcastle virus to complete the cross-immunity tests, but to wait until such a virus could be freshly collected from the field. An outbreak occurred at one of the Ministry's breeding farms at Sakha in April, 1947, but unfortunately it had progressed too far for effective intervention before it was reported to this laboratory, since all but 300 of the 7,329 birds on the farm were already infected. On the assumption that the disease would prove to be classical fowl-plague, an attempt was made to immunise a number of birds in uninfected pens with tissue-vaccine and with a formolised aluminium gel-adsorbate vaccine prepared from a chick-embryo cultivated strain of fowl-plague virus, but within seven days of inoculation all the birds on the farm, except 40, were dead. In the meantime, a strain of virus had been cultivated in the chick-embryo from birds sent to the Institute from Sakha, and immunity tests and red-cell agglutination-inhibition tests had shown it to be a highly virulent Newcastle strain (Tables II and III). The final number of survivors from the outbreak was 27 and the duration of the epizootic 17 days. Roughly 40 days later, 100 susceptible birds

were introduced into the pens and allowed to mix with the 27 survivors. There was no further outbreak among these susceptible birds. It might be remarked in passing that these breeding farms of the Ministry, of which there are seven carrying in all about 30,000 to 35,000 birds, offer an excellent opportunity to make carefully controlled tests of vaccines or other prophylactic agents.

A month later, another outbreak of suspected fowl-plague was reported from the Ministry's poultry farm at the Barrage, which

TABLE II

IMMUNITY TESTS WITH SAKHA VIRUS

Status of birds	*Number*	*Description*	*Challenge*	*Result*
Immune to fowl-plague	2	Chicks, six weeks old	1 c.c. 10^{-1} 2nd passage, Sakha, Allantoic fluid	D3, D12*
„	1	„	Contact only	D7.
Susceptible controls	3	„ old	1 c.c. 10^{-1} 2nd passage, Sakha, Allantoic fluid	D2, D3, D3.
Immune to Newcastle disease	2	Adult birds	1 c.c. 10^{-3} 2nd passage, Sakha, Allantoic fluid	Both survived
Immune to fowl plague and Newcastle disease	2	„	„	Both survived
Susceptible controls	1	Cockerel, 4 months old	1 c.c. 10^{-3} 2nd passage, Sakha, Allantoic fluid	D3.

* This bird was ill 42 hours after inoculation, but by the seventh day it had apparently recovered. It relapsed on the ninth day.

TABLE III

CCA INHIBITION TESTS WITH SAKHA VIRUS

Serum	*Antigen (Allantoic fluids)*	*Serum dilutions* 1 : 25	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	*Control No serum*
Newcastle immune serum	Sakha, second passage	o	o	o	o	o	Not done	++++
	Sakha, sixth passage	o	o	o	o	o	o	++++
	Fowl plague, "A" 43rd	+	+	+++	+++	+++	Not done	+++
	Fowl-plague, Strain R, second passage	++++	++++	++++	++++	++++	++++	++++
	Newcastle vaccine strain	o	o	o	o	o	o	+++

at the time carried roughly 5,300 gallinaceous birds. The existence of the disease was suspected in a pen, No. 24, situated near the northern boundary of the farm, on May 8th. On May 10th the birds from this pen—400 in number—were removed to a quarantine enclosure just outside the southern boundary. On May 17th a second pen, No. 21, also on the northern boundary, became infected : the birds from this pen, 410 in number, were also removed to the quarantine enclosure on May 18th. All birds in the other pens were confined to the houses from May 10th onwards. The next pen to become infected was pen No. 22 (90 birds), where a death occurred on May 17th. On May 23rd, pen No. 23 (260 birds), adjoining pen No. 24, showed infection, and finally, on May 29th, pens Nos. 11 and 12, containing 453 young birds, two months old, became infected.

A diagnosis of Newcastle disease was established by inoculating spleen and heart blood into the chorio-allantoic cavity of embryonating eggs, collecting allantoic fluid from the eggs at death of the embryo (42 hours in the case of those inoculated with spleen, and 72 hours for the heart-blood inoculation), and finally testing the capacity of a known Newcastle-immune serum to inhibit chick-cell agglutination by the allantoic fluids from infected eggs. Table IV shows

TABLE IV

CCA INHIBITION TEST WITH SAKHA AND BARRAGE STRAINS OF VIRUS

Serum	*Antigen (Allantoic fluid)*	*Serum dilutions*						*Control*
		1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1,280	
Newcastle immune serum	Sakha 8th 1 : 80	o	o	o	o	o	++++	++++
	Barrage 1st 1 : 80	o	o	o	o	±	++++	++++

the result of the first test made within 42 hours of the receipt of sick birds for diagnosis. A further test was made five days later. The antigen was Sakha virus (eighth passage), and several sera were tested for inhibitory power. The pooled sera of four sick birds brought from the Barrage farm only three days previously failed to inhibit agglutination in dilutions from 1 : 20 upwards ; sera from two birds brought to the Institute on May 11th, and apparently recovered by the 17th, inhibited in one case up to a dilution of 1 : 1,280, and in the other up to a maximum of 1 : 640. Susceptible birds, including small chicks, inoculated with material used to infect chick-embryos did not begin to die until the seventh day, *i.e.*, five days after a diagnosis had been obtained by the chick-embryo inoculations.

Meanwhile, some of the birds on the farm had been inoculated with a formolised aluminium-gel adsorbate, chick-embryo vaccine

against fowl-plague, and an abortive attempt had been made to immunise others with stored Newcastle vaccine which on test proved not to be viable. Apart from the removal of the birds from pens Nos. 24 and 21 to the quarantine enclosure, the confinement of the remaining birds to the houses and the application of commonsense precautions regarding the allocation of attendants, utensils, and general disinfection, there was no effective intervention against the disease until May 23rd, when inoculations with a fresh batch of Newcastle vaccine were begun. The first pens to be inoculated were Nos. 22 and 23, where infection was already evident, and by May 26th all fowls and turkeys had been injected with vaccine, including the birds in the quarantine. Table V summarises the data for the six infected pens, in respect of deaths before and after vaccination with the Muktesar strain.

TABLE V

MORTALITY IN INFECTED PENS AT THE BARRAGE FARM BEFORE AND AFTER VACCINATION WITH MUKTESAR STRAIN

Pen No.	*Number of birds*	*Interval between detection of infection and vaccination*	*Before immunisation*		*After immunisation*	*Survivors*
			Deaths	*Taken for diagnosis and experiment*	*Deaths*	
24	400 *	18 days	363	52	95	305
21	415 *	8 „				
22	95	1 day	0	26	19	50
23	260	0 „	0	1	42	217
11, 12	453	—4†	0	0	65	388

* Pens mixed in quarantine.

† First case four days after vaccination. Possibly post-vaccination paralysis.

Fig. 1 is a histogram showing the mortality for the months of May and June, 1947, grouped for convenience in periods of three consecutive days. The figures include all deaths on the farm, *i.e.*, they represent the normal mortality plus the deaths from Newcastle disease. It will be observed that deaths reached a peak during the period May 22nd to 24th, the highest daily mortality being that for May 24th, *viz.*, 108. Immunisation, from May 23rd to 25th, appeared to effect a rapid reduction in the infection and mortality rates at a time when infection had just become established in two fresh pens on two consecutive days. It is doubtful whether the mortality that occurred among the two-months-old chicks of pens Nos. 11 and 12

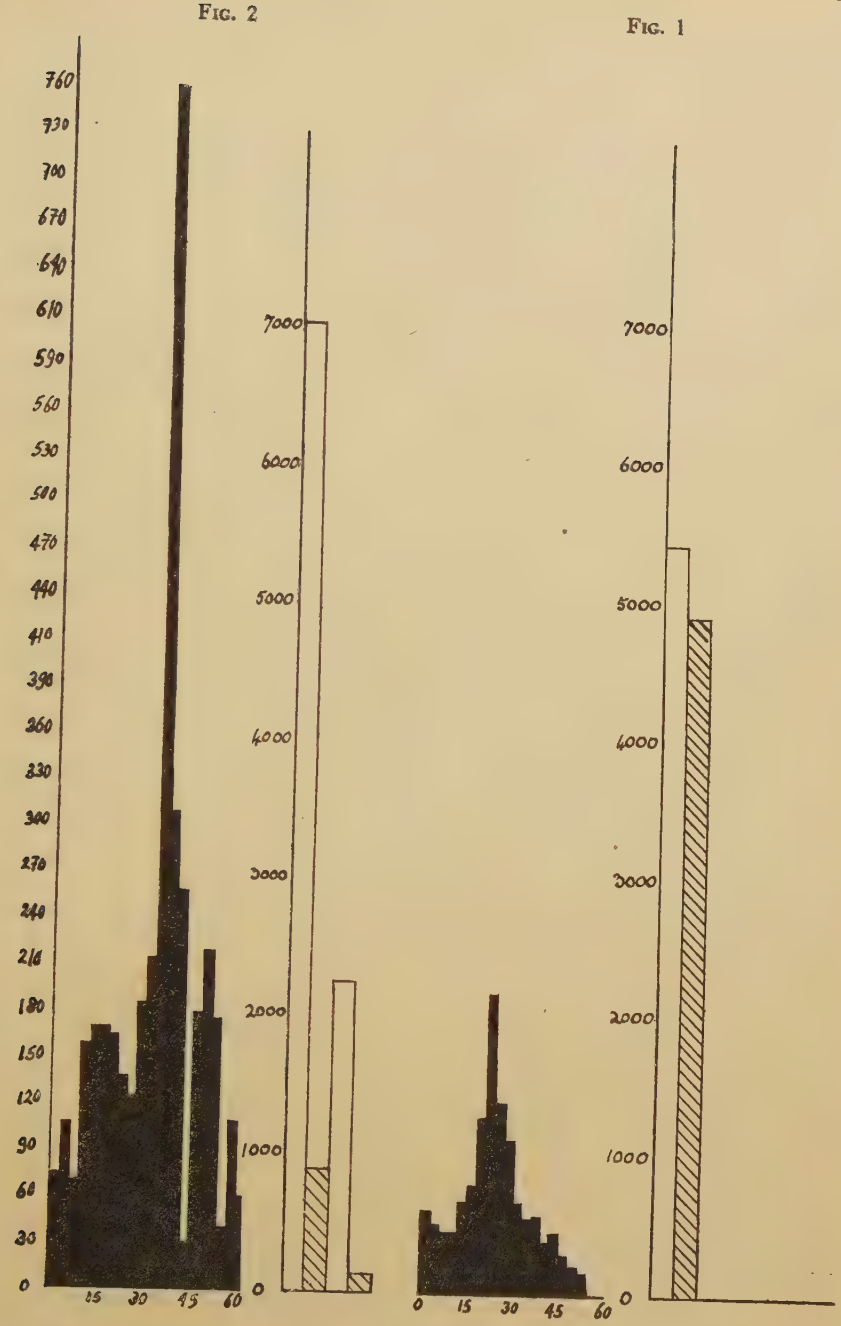

Fig. 1

Mortality for months of May and June in the 1947 outbreak at Barrage Farm, grouped in 3-day blocks. Immunisation with the Muktesar strain was carried out on the 23rd, 24th and 25th days, when the epizootic was at its height. The block diagram at the right compares the total population (plain) with the survivors (hatched).

Fig. 2

Mortality for months of April and May, 1946, covering a previous outbreak at Barrage Farm. Deaths are grouped in 3-day blocks as in Fig. 1. In the block diagram at the right, the first pair of blocks indicates total initial population (plain) and survivors (hatched). The second pair of blocks represents total vaccinated with tissue vaccine (plain) and survivors (hatched) from the vaccinated groups.

between the fourth and eighth days after inoculation was due to post-vaccination paralysis or to natural infection ; unfortunately, no specimens were submitted to the laboratory. Eighteen cases of paralysis were observed in two pens (Nos. 18 and 20) containing 950 adult birds ; those paralysed birds that were weak and emaciated died, but the majority recovered and no cases remained at the 15th day. Of the 5,323 birds alive on May 15th, 4,748 survived on June 26th. The two pens of quarantine birds were returned to the farm proper on June 7th, and it is believed that the last deaths from the disease, those in pens Nos. 11 and 12, occurred on June 2nd, seven days after the completion of vaccination.

During the last ten years almost every one of the Ministry's farms has experienced similar outbreaks of disease in, say, two out of three years and a brief reference might be made to a previous outbreak of what appears to have been the same disease on this particular farm in 1946. Deaths began to occur about April 1st, when there were 6,829 birds on the farm. Fig. 2 is a mortality histogram for the months of April and May, 1946 compiled from the farm records. Deaths are grouped in three-day periods as in Fig. 1. In spite of the slaughter of 2,246 birds in attempt to control the spread of infection, there were only 811 survivors at the end of May. Between January 29th and April 21st, 2,098 birds of four flocks had been vaccinated twice with tissue-vaccine, and in every pen, except one, the second dose had been received before infection was diagnosed in that particular flock. At the end of May, however, there were only 125 survivors, which is about half the number expected in a chance distribution of survivals. Symptoms and lesions are said to have resembled those of the recent outbreak, and a tentative diagnosis of infectious laryngo-tracheitis or of a new strain of fowl-plague was made.

Since it had now been demonstrated that the first two outbreaks of so-called fowl-plague to occur on the breeding farms in 1947 were in fact due to the virus of Newcastle disease, it was decided to vaccinate all the Ministry's birds with the Muktesar-Newcastle vaccine, as well as with an aluminium-gel-adsorbate vaccine against fowl-plague. Accordingly, in May, June and July, roughly 30,500 birds on six farms were vaccinated. Turkeys and guinea-fowls were vaccinated as well as fowls, and the youngest chicks treated were about 35 days old. Ducks and geese were not inoculated.

Reports indicated that a few cases of paralysis were observed after Newcastle vaccination in adult birds and in three to four months old chicks, particularly where these were in poor condition or affected with chronic lesions of fowl-pox, but most of the affected birds recovered. There was, however, a definite mortality from post-vaccination paralysis in very young chicks. To take the figures from one farm, Inshas as an example, the mortality for two periods of 18 days, the one preceding and the other following vaccination, is set out in Table VI. It will be observed that there was no increase in

TABLE VI

MORTALITY ON INSHAS FARM (NOT INFECTED) DURING PERIODS OF 18 DAYS BEFORE AND AFTER VACCINATION WITH MUKTESAR STRAIN

Breed	*Age*	*Total Inoculated*	*Mortality 18 days preceding vaccination*	*Mortality 18 days following vaccination*
Egyptian birds	Adults	1,263	27	31
	Chicks	10,790	700	599
Rhode Island Reds	Chicks, 37 days	300	3	9
Plymouth Rock	„ 35-40 days		3	2
Light Sussex	„ 35-40 „	300	1	36
Australorps	„ 35-40 „		3	37
Leghorn	„ 35-40 „	550	56	85
Minorca	„ 35-40 „	240	5	2

mortality of adult birds or chicks of Egyptian breeds, nor in very young chicks of the Rhode Island, Plymouth Rock or Minorca birds, but a significant increase among young Light Sussex, Australorp and Leghorn chicks. The sick chicks showed the usual symptoms of paralysis, but on *post-mortem* examination none of the characteristic lesions of Newcastle disease could be found. It was not possible to recover the virus from the blood, liver or spleen either by chick or chick-embryo inoculations, but further investigations must be made before it can be asserted that the modified virus cannot infect by contact. Instructions have now been given that chicks should not be vaccinated until they reach the age of two months.

With regard to the immunity evoked by the Muktesar vaccine strain, it may be of advantage to tabulate some of the tests made with vaccinated and control birds. Reference to Table VII will show that 72 birds, immunised with various batches of vaccine at the Institute and on two of the farms between June 1st and 27th, were challenged with virus, and all survived, whereas 20 birds, each treated three times with tissue-vaccine prepared from artificially infected birds, and eight uninoculated controls, all succumbed to the test inoculation. In a second test, 76 birds, vaccinated at Dokki on 1.6.47, were challenged with 1 c.c. $\times 10^{-3}$ of Bacouse strain allantoic fluid (fourth passage) on 2.12.47, *i.e.*, six months after vaccination. None died or showed any symptoms of illness after the test, while of three control birds bought in the open market one died on the sixth day, one on the seventh, and the third bird proved to be immune.

Later tests have included 146 birds from the Barrage farm, and 30 from the Gemeza farm, challenged at intervals of 331 to 359

TABLE VII

IMMUNITY TESTS OF BIRDS VACCINATED WITH NEWCASTLE VACCINE (MUKTESAR STRAIN)

Number of birds	*Place and date of immunisation*	*Interval between immunisation and challenge*	*Test dose*	*Result*
44	Dokki, 1.6.47	66 days		All survived
8	Abbassiah, 27.6.47	40 „		„
20	Inshas, 11.6.47	56 „	Allantoic fluid,	„
5	Dokki, 1.6.47	116 „	Bacouse strain,	„
10	Treated tissue-vaccine (spleen) 3 doses, 5.5.47 to 19.5.47	Regarded as susceptible controls	Passage IV, 1 c.c × 10^{-3}	All died on 6th-8th days
10	Treated tissue-vaccine (liver), 3 doses, 5.5.47 to 19.5.47			„
8	Susceptible controls bought in open market	—		„
76	Dokki, 1.6.47	184 days	Allantoic fluid, Bacouse strain,	All survived
3	Susceptible controls bought in open market	—	Passage IVB 1 c c. × 10^{-3}	D6, D7 One survived
46	Dokki, 1.6.47	353 days		
30	Gemeiza, 23.6.47	331 „	Allantoic fluid,	
50	Barrage, 26.5.47 (More than 1 year old when vaccinated)	359 „	Bacouse strain, Passage XIV 1 c.c. × 10^{-3}	All survived
50	Barrage, 26.5.47 (From 1 to 7 months old when vaccinated)	359 „		
10	Gemeiza. Controls 6 months chicks	—		D5, D5, D6, D6, D6, D6, D6, D6, D6, D6

days after vaccination. Fifty of the Barrage birds, tested at 359 days, were from one to seven months old when vaccinated, but they appear to have developed a satisfactory immunity as a result of the protective inoculation. During the period between vaccination and testing, no cases of Newcastle disease were detected on either the Barrage or the Gemeza farms, but on the Barrage farm there were no unvaccinated birds. On the Gemeza farm there were several hundred unvaccinated birds that were less than three months old at the time of the general vaccination.

One would expect treatment with a modified living virus to give protection to birds very soon after inoculation, and the record of the outbreak on the Barrage farm lends support to the view. Moreover, it was observed at the time that vaccination of birds in the early

stages of the naturally contracted infection was frequently followed by recovery, particularly when the birds were given two to three times the normal dose of vaccine. That the " interference phenomenon " is concerned, is well illustrated by the result of a small experiment in which birds were challenged at intervals ranging from one day (24 hours) to eight days after vaccination. One bird only was challenged on each day, and a single susceptible control was included, together with three birds that had been treated with three doses of vaccine prepared from the organs of infected adult birds. The control bird showed symptoms on the sixth day and died on the eighth day ; the tissue-vaccinated birds all showed symptoms on the fourth day and died on the seventh day ; the Muktesar-vaccinated birds challenged on the first, second and third days after vaccination showed slight symptoms of illness from the seventh to the eleventh days and recovered ; and the remaining birds, challenged four, five, six, seven and eight days after vaccination, showed no ill effects.

In another experiment an unsuccessful attempt was made to demonstrate the curative value of vaccination on already infected birds. The birds, 24 in number, were infected by the intramuscular injection of 1 c.c. $\times 10^{-3}$ dilution of allantoic fluid of a virulent strain, and were subsequently treated by injecting groups of eight with 1 c.c. of 1 : 10 dilution of allantoic fluid of the Muktesar vaccine-strain 24, 48, 72 and 96 hours later. All died within the usual period for susceptible birds inoculated with the virulent strain. The result was disappointing in view of the clinical experience referred to above, but possibly the initial infecting dose of virus, which represented at least 10^6 infective units, was too high.

The vaccine is easily prepared and the allantoic fluid from a single embryo will furnish some 4,000 to 5,000 doses. In our hands, the titre of allantoic fluids at death of the embryos (42 hours) ranges from $10^{7 \cdot 5}$ upwards, depending possibly on the state of dispersion of the virus. The allantoic fluids can be desiccated for purposes of transport and storage with the usual loss of titre.

SUMMARY

It has been shown that Newcastle disease undoubtedly occurs in Egypt and that it can be responsible for enormous losses. Under the conditions that obtain on large poultry farms, outbreaks can be rapidly suppressed by the use of the Muktesar vaccine-strain of the virus which, however, has well-marked neurotropic affinities. In Egypt, a serious incidence of fatal post vaccination paralysis has been observed only in very young chicks, but there is some evidence that intercurrent infections such as fowlpox, may increase the incidence of paralysis in slightly older birds.

There is good reason to believe that the vaccine strain of the virus multiplies rapidly in the inoculated susceptible bird and that, in consequence, inoculated birds are soon protected by " interference."

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Footnote.—The authors regret that when the present note was prepared they were not aware of the fact that an account of the original work with the Muktesar strain of virus had been published in the *Indian Journal of Veterinary Science, vide* Dr. J. R. Haddow's letter of June 24th, 1947, published in *The Veterinary Record* July 5th, 1947. Neither were they aware that any account had appeared of the use of the vaccine in Palestine, until the publication in *The Veterinary Record* of August 9th, 1947 of Mr. Chana Aheroni's letter of July 27th, 1947. At the time of writing, neither of the publications had been available to the writers for consultation.

PATHOGENESIS OF BLACK QUARTER

I. Tissue Damage and Spore Latency

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INTRODUCTION

The problem of the pathogenesis of black quarter has exercised the imagination of many observers and may be one of much practical importance. As Heller (1920) has remarked, " epidemiologic factors in anaerobic infections are of most interest, and their demonstration would prove a most valuable contribution to science and to agriculture." An outstanding and well-known fact is that, while a few *Cl. chauvoei* organisms inoculated into the muscles of susceptible animals usually produce a rapidly fatal infection, massive quantities given by the mouth have no obvious effect. Since, in cattle at least, no surface wound which may act as the portal of entry is usually to be found, it is generally believed that spores entering by the mouth find their way into the tissues and are enabled to germinate through some interference with the normal tissue equilibrium.

In India, black quarter tends to occur at about the time of the summer monsoon. The reasons for this are not clear, and it is not known whether the parasite or the host, or both, are concerned. Thus, it can well be imagined that spores present in the surface layers of the soil become more readily available to susceptible animals as a result of rain. Bodily resistance may be lowered in some way, possibly owing to the cooling effect of rain showers, or the improvement in bodily condition which comes with the monsoon may for some reason increase susceptibility. It will be remembered that fairly young animals in good condition are most liable to be affected in nature.

Some few facts concerning these problems have been collected at this Institute during the past few years and are recorded in three papers. Guinea-pigs and sheep have been mainly used as experimental animals, but the results would probably be applicable to cattle. This first paper deals with the varying susceptibility of young sheep apart from possible seasonal causes, the question of local tissue damage and the latency of *Cl. chauvoei* spores in living tissues.

PREPARATION OF SPORE SUSPENSION

The first problem was to prepare a suspension of spores in which the numbers of viable organisms would remain reasonably constant.

The strain of *Cl. chauvoei* in use throughout this work is one which readily forms spores and which has been subjected to relatively few

subcultures. Cultures were prepared in a medium of beef-heart mash in liver broth, left for two or three days at 37° C. and then at room temperature for two more days. The culture was stirred up to separate the organisms as far as possible from the meat particles. After the larger particles had settled, the supernatant fluid was centrifuged and the deposit, washed once in distilled water and well shaken in a flask with beads to break up clumps, was taken up in an arbitrary amount of distilled water, usually about 20 c.c. to the product from 12 tubes (15 × 1·8 cm.) of medium. The suspension was heated at 70° C. for 30 minutes and stored in the cold (±5° C).

Twenty suspensions were prepared in this way at various times. The first six gave counts by the Breed smear method of about 4 to 13 millions per c.c. With eight of the last suspensions prepared, titrations were made using the probability tables of Halverson and Ziegler (1933), five tubes of meat-liver medium being seeded with 1·0 c.c. of every tenfold dilution covering the end point. The suspension by this method gave counts varying from 7 to 49 million per c.c., and was not dissimilar in richness to a meat-liver culture grown for 48 hours at 37° C. The meat-liver medium was put up in small culture tubes (12·5 × 1·4 cm.) containing about 9 c.c. medium, of which not less than three quarters of the volume consisted of meat mash. Provided the medium is made in this way, it appears to be fairly suitable for titration purposes, though growth is apt to fail in a proportion of tubes about the end-point. Before each titration the spores were brought into uniform suspension by thoroughly shaking the tube.

When the number of spores used in an experiment is stated, the figure will have been obtained from a Breed smear or from the five-tube count, and can be little more than a rough estimate. In some comparative experiments, the purpose is served by giving the dilution used.

The germination of spores after storage in the cold for a week or ten days is retarded, compared with spores stored at room temperature, when they are brought to favourable temperature and nutrient conditions. This increase in germination time is important because if such delay occurred *in vivo* the opportunity for phagocytic action would be increased and infection might be prevented. In a single experiment to test this hypothesis the result was clear-cut in its favour.

Two experiments were carried out to see the effect on suspensions of prolonged storage—upwards of 800 days—in the cold. Under these conditions the titre of distilled water suspensions was found to have declined to some two-thirds of the initial, perhaps due to some spontaneous clumping. But when the spores were suspended in 40 per cent. glycerol and M/100 phosphate, *p*H 7·2 (suspension 50, M/10 phosphate 10, glycerol 40) the titre fell to one-third or less of the original. Judging by the opacity of the suspensions, the effect of glycerol was to dissolve or render more transparent the bulk of the spores.

One important point is that the water used for making the suspension should be glass-distilled. On one occasion the viable spore

count fell greatly on storage and it was then found that copper-distilled water had been used. The copper content of this water was found by Dr. N. D. Kehar to be 11·2 p.p.m.

ROUTES OF INOCULATION

After titrations *in vitro,* suspensions were tested intramuscularly on young sheep, usually yearlings, several dilutions at 0·5 or 1·0 c.c. dose being injected into the muscle at the back of the thigh. For the purpose of experiments in view, it was hoped in this way to establish the potency of suspensions in terms of numbers of spores able by themselves to set up infection. This, however, was not possible owing to the variation in susceptibility. One example will suffice, though similar instances are mentioned later.

A suspension, prepared on 27.6.42, when titrated a month later, contained about 7 million spores per c.c. It was used in a dose of 0·5 c.c. intramuscularly (Table I).

TABLE I

Number of sheep inoculated	*Date*	*Dilution*	*Result*
1	27.6	10^{-1}	+
1	27.6	10^{-2}	+
6	16.7	10^{-3}	3 + 3 −
4	2.9	10^{-3}	3 + 1 −
8	18.7 to 21.7	10^{-4}	5 + 3 −
6	6.8	$10^{-4.3}$	2 + 4 −
4	29.7	10^{-5}	4 −

+ = died — = survived

Thus, of 24 sheep injected at 10^{-3} to $10^{-4.3}$ (3,500 to 116 spores), 13 succumbed with typical lesions of black quarter, but the rest were unaffected. Thirty-five spores failed to infect four sheep, but it was found that this number of spores from the same suspension, given intramuscularly, infected one out of 12 guinea-pigs.

Under such circumstances there is little point in trying to establish a minimal infective dose of black quarter spores for sheep, at least by the intramuscular route. There is some evidence that the result of animal inoculation tests depends not only on the number of spores but also their concentration in the fluid inoculated. Thus, in one experiment spores suspended in 0·1 c.c. saline were infective, but the same number of spores in 1·0 c.c. was not.

Specific reference in the literature to the variable susceptibility of animals to Clostridia has been noted. Coleman and Meyer (1922) encountered the phenomenon in guinea-pigs injected with *Cl. botulinum* spores, but they do not try to explain it. Scott (1924-25) was troubled by a similar variation in experiments with *Cl. chauvoei* in guinea-pigs, though apparently the better-fed animals were considered to be more resistant.

In general, susceptibility appears to be lower by the subcutaneous than by the intramuscular route, as would be expected from the fact that the oxygen tension in the subcutaneous tissues is higher than that in resting muscle (Campbell, 1924-25). Thus in one experiment five sheep were injected intramuscularly and five injected at the same time with the same or larger doses subcutaneously. Of the former, four promptly died of black quarter; of the latter, only one became infected and then only after a week.

There is little danger of infecting by the intravenous route. Quite frequently, millions of spores have been injected into the blood without producing infection, though occasionally lesions develop in the subcutaneous tissues of the neck about the site of inoculation.

There are two other points to be noted. First, whereas in sheep inoculated intramuscularly the lesion is usually apparent within two days and death ensues during the following 24 hours, occasionally the lesion does not appear for a week and at other times the animal slowly recovers with much shedding of wool and sloughing of skin. Such recovery is not at all uncommon even after calcium injections. Secondly, after intramuscular inoculation at the back of the thigh, lesions occasionally develop at some distant part of the body, *e.g.*, in the subcutaneous tissues under the neck or sternum or in a fore limb, illustrating the drift of organisms which takes place from the site of inoculation. A similar fact has been noted by Gaiger (1924) following subcutaneous inoculation of *Cl. septicum* in sheep.

LOCAL DAMAGE IN RELATION TO PATHOGENESIS

Since it is well-known that experimentally a number of substances, such as calcium chloride and lactic acid, function as activators or tissue depressants and, through a lowering of oxygen tension, enable latent spores to germinate, the question has been raised whether depressant action under natural conditions might be brought about in the absence of wounds in various ways, *e.g.*, by pathogenic organisms, by blood clot or by damage to muscle fibres from accumulated lactic acid. In "black disease" of sheep due to *Cl. oedematiens*, necrotic areas in the liver resulting from migration of flukes permit germination of latent spores (Turner, 1930). A few experiments were done with blood clot and on liver damage.

Blood clot

It is possible that spores entangled in blood clot find conditions suitable for germination. Two satisfactory experiments were made *in vitro* to see if spores would germinate in clot as readily as in meat-liver medium.

In the first experiment, falling numbers of spores were sown in (*a*) tubes of meat-liver medium, and in (*b*) blood clot, submerged in sterile broth. In the former case, the medium was exposed to the air as usual,

while the blood clot tubes were incubated in an anaerobic jar. In setting up the blood clot medium, 1·0 c.c. of serial dilutions of spore suspension were placed in culture tubes (each containing a glass bead), mixed with 0·5 c.c. sterile citrated sheep blood. A minimal amount of $CaCl_2$ was added to produce coagulation (about 0·05 c.c. 1·0 per cent. solution) and the clot was then lacerated and covered with 8·5 c.c. broth. Controls (*c*) without $CaCl_2$, and (*d*) with saline in place of citrated blood were included. Some amount of haemolysis of the blood or blood clot appeared to be a fairly satisfactory indicator of germination. The result of the experiment was that germination occurred with fewer spores in meat-liver (to 10^{-6}) than in clot (to 10^{-4}). Growth took place in (*c*) to 10^{-4}, and no growth in (*d*).

This experiment was repeated with the modifications that the blood-spore mixture in the tubes was exhausted before and not after clotting, and that no broth was added to the tubes. The tubes containing the mixture were put in an anaerobic jar, the jar exhausted and then tilted so that the blood mixture came in contact with a small packet of filter paper containing $CaCl_2$ suspended near the plug. Clotting was then rapid. In this experiment also germination in meat-liver went to 10^{-6} but growth was proved by subculture to have occurred in clot (duplicate tubes) and in unclotted blood (control) to 10^{-4} only.

The results of three experiments *in vivo*, likewise gave no suggestion that blood clot is specially favourable for germination. In Experiment 1, blood from the jugular vein was mixed with spore suspension in a tube and quickly injected subcutaneously into the same animal. In a control sheep, saline in similar amount was substituted for the blood. Neither animal reacted. A few days previously, two sheep had been injected subcutaneously with the same suspension but with four times the number of spores used in the blood-clot experiment, and both developed black quarter.

In Experiment 2, three sheep were inoculated with a suspension diluted at 10^{-4} 10^{-3} and 10^{-2}, respectively, 1·0 c.c. of the particular dilution being injected into the muscle at the back of each thigh. Immediately beforehand, the muscle of the one thigh was injured over an area of one-inch diameter by repeatedly thrusting in a needle the point of which was slightly bent back. The inoculation of spores was made into the middle of this injured area. No infection ensued. (During previous days, infection had been set up with the same suspension by simple intramuscular inoculation—in two of six sheep with 10^{-4}, in one of three with 10^{-3} and in one of two sheep with 10^{-2}.)

Finally, in Experiment 3, two sheep were injected intravenously with 5·0 c.c. spore suspension (at 10^{-1} ; numbers unestimated) ; 20 minutes later the muscle of one thigh was lacerated by a dozen needle thrusts, and three hours later the other thigh muscle was similarly treated. No infection occurred.

Liver damage by Carbon tetrachloride

In principle, the chances of setting up black quarter in this way might seem to be favourable in the light of Turner's work and that of Edgar (1928). The experiments, however, were negative, possibly because time relationships are important and the proper conditions were not realised, but more likely because the oxygen tension in

slightly damaged liver tissue could not be maintained at a sufficiently low level.

According to White (1939), in swine the drug in a dose of 0·6 c.c. per kg. causes considerable liver destruction. To fix a suitable dose of CCl_4 for the work with sheep, three animals weighing 15, 14 and 27 kg., were given the drug subcutaneously at two points over the abdomen at doses of 0·3, 0·6 and 0·9 c.c. per kg. respectively. The first and third sheep were "drunk" for a few minutes immediately after the injections. All were killed by bleeding, the first two 96 hours and the third 53 hours after injection. Their livers showed no obvious change macroscopically. Microscopically, the changes were: 0·3 c.c. dose, nil; 0·6 c.c. dose, the fringes of the lobules stained more intensely with chromotrop 2R than the centres, *i.e.*, towards the centres there was some loss of cytoplasmic and possibly of nuclear staining; 0·9 c.c. dose, definite loss of liver cells at centres of lobules. In another sheep, killed seven days after the 0·9 c.c. dose, the cells at the centres of the lobules were swollen and stained rather deeply with eosin.

Ad hoc experiments were then put up, 0·9 c.c. per kg. being adopted as the standard dose of drug and 5·0 c.c. intravenously as the volume of spore suspension. (*a*) Two sheep (11 and 14 kg.) were given the suspension 24 and 48 hours before the drug, and three sheep (12·7, 11 and 15 kg.) were injected at 0, 24 and 48 hours after the drug. The numbers of spores for the five sheep in question were: unestimated, 2·2, 5·0, 5·0 and 0·35 millions. No infection resulted. (*b*) A sheep (22 kg.) was given a large dose of spores intravenously and immediately after CCl_4 subcutaneously. Three hours later the injection of spores was repeated. Four days later CCl_4 was again given and three hours afterwards a third injection of spores. The sheep remained alive and well.

DEMONSTRATION OF LATENT SPORES

There are many references to the latency of the spores of anaerobic bacteria. Koser and McClelland (1917) recovered *Cl. tetani* from the liver and spleen of rats up to two months after subcutaneous injection. In similar experiments, they recovered "*B. anthracis symptomatici*" from the liver for a month and from the spleen for two months. Basset (1925) isolated black quarter spores for at least 12 days from the healthy tissues of inoculated guinea-pigs. Turner (1930) recorded *Cl. oedematiens* spores for as long as nine months from the liver and spleen of sheep inoculated by various routes, and apparently such carriers are quite common in Australia.

Spore latency may be demonstrated (*a*) by means of tissue depressants, (*b*) by recovery and identification of spores after death.

Use of tissue depressants

Calcium chloride (0·5 c.c. of 5 per cent. solution) has been mainly used in this work, in many cases for sheep which, after injection of spores, have been subjected to some other treatment such as chilling of the body. Lactic acid (2·5 per cent.) and arsenious oxide (5·0 mg. per c.c.) were also tried. In the event of sheep not reacting to the

treatment, $CaCl_2$ has been injected more or less as a routine at the site or sites of the original spore inoculation to see if spores still persist. In this case they germinate at the site and set up black quarter.

By this means spore latency after *subcutaneous* inoculation in the loose tissue of the back or behind the thigh has frequently been demonstrated for 15 days and on two occasions for 25 and 40 days. Altogether, out of 41 tests for spore survival in subcutaneous tissues, 23 have been positive and 18 negative. Of the latter, however, 14 were tests in the subcutaneous tissue about the fetlock, while of the 23 positive tests only three resulted from tests in this region. This shows that the spores have little tendency to persist in the tighter connective tissues at the distal parts of the limbs. It has been known for years that the black quarter oedema tends to develop in regions of the body where there is much loose connective tissue and not in situations such as the extremities of the limbs and the tail where the temperature is somewhat lower and the connective tissue minimal, *e.g.*, ear-tip and tail-tip methods of vaccination. Ten out of 23 tests, in which spores had been deposited in *muscular* tissue were positive: three were in sheep injected 20 and 25 days previously and in the remainder the period was shorter. Examination of all the results obtained has failed to show that persistence is correlated with the number of spores injected. After intravenous injection calcium has been injected intramuscularly on a number of occasions at intervals up to 14 days. In three of these sheep, the calcium was injected at numerous points in the muscle, first of one thigh and then of the other, 20 minutes and three hours after the spores. Only once—after at least 50 million spores had been injected—was the existence of spores in the muscle shown, *viz.*, in a sheep injected 20 minutes after the spores had been given.

Recovery of spores by cultural methods

Attempts to recover black quarter spores after death have been made on ten sheep, either from animals which after inoculation have shown no symptoms or, as occasionally happens in normal sheep, from those which have survived an experimental attack. The results are shown in Table 2.

Sheep 6 became very thin and was killed when moribund on the 25th day. Sheep 7 had a clinical attack which was nearly fatal within three days, but recovery was then rapid. Sheep 8 had recovered from a clinical attack. Sheep 9 as a result of previous inoculations evidently had a good immunity. This sheep was killed 32 days after being inoculated with a heavy dose of spores plus Ca. chloride which however produced only fever and transient local swelling. Sheep 10 recovered from black quarter in one hind limb, accompanied by much necrosis of skin and subcutaneous tissue.

The technique was to kill the animal by bleeding, at once remove samples of the various tissues, mash up pea-sized bits (after flaming their exterior) in sterile tubes containing about 3 c.c. saline after first chopping

the material with scissors, sow meat-liver tubes from the saline suspension, in the unheated state and again after heating at 70° C. for 30 minutes. Cultures showing gas on incubation, and more especially those from the heated suspensions, were examined and, if organisms resembling *Cl. chauvoei* were present, the culture was again put at 70° C. for 30 minutes and sown into meat-liver and into an aerobic control medium. Cultures then presumed to contain *Cl. chauvoei* were mixed with some Ca. chloride and inoculated into healthy and *chauvoei*-immune guinea-pigs (Minett, 1945). This gave a very satisfactory differentiation. Sometimes, the type of colouy developing on blood-agar slants in an anaerobic jar was noted.

In all cases shown as positive in the table, the cultures were identified by the immunity test. The following tissues were examined. Organs; liver, spleen, kidney (less commonly), bone-

TABLE II

Sheep number	*Inoculated*	*Symptoms*	*Killed on day*	*Result*
1	Intrav.	None	2	Spleen pos.
2	„	„	6	B. marrow pos.
3	„	„	15	Popliteal gland pos. (Culture impure.)
4	„	„	15	Negative.
5	„	„	22	„
6	Intramusc.	„	25	B. marrow pos.
7	„	Bl. quarter	8	Negative.
8	„	„	15	B. marrow neg.
9	„	„	32	Spleen and sublumbar glands neg.
10	„	„	18	B. marrow, sublumbar and mediastinal glands pos.

marrow (head of femur): glands; pharyngeal, prepectoral, bronchial, mediastinal, mesenteric, hepatic, sublumbar and popliteal. Contaminated tubes were common and at times some trouble was experienced in isolating the organism; other anaerobes were also encountered. Sheep 4 and 5 were exceptional in that most of the culture tubes showed no growth, even from the unheated suspension. In some of the cases shown as negative, organisms were isolated which somewhat resembled *chauvoei* but whose identity was not established. Again, some of the findings classed as negative might have been positive on more intensive examination, but no attempt was made to set up records. The results show recovery of *Cl. chauvoei* from spleen after two days, from bone-marrow after 6, 18 and 25 days, from gland tissue after 15 and 18 days.

SUMMARY

Young sheep, inoculated intramuscularly with black quarter spores suspended in distilled water and stored in the cold, vary so much in susceptibility that it is not feasible to fix a minimal infec-

tive dose. There was some evidence of greater susceptibility by the intramuscular than by the subcutaneous route.

Large numbers of spores given intravenously have no obvious effect. Sheep experimentally infected usually die within two or three days. Occasionally, the lesion does not appear for a week and at times may not occur at the point of inoculation but at some distant site.

In connection with the nature of the "local damage" which permits latent spores to germinate, there was no evidence from *in vitro* or *in vivo* experiments that blood clot assists germination: nor could it be shown that liver injury by carbon tetrachloride assists germination of spores given intravenously.

By means of calcium chloride, spore latency has been demonstrated up to 25 and 40 days in subcutaneous tissue, and up to 20 to 25 days in muscle tissue.

By cultural methods, spore latency has been determined for periods up to 25 days in bone-marrow and up to 18 days in lymph gland. In these cases the sheep had either shown no symptoms after the original inoculation of spores or had recovered from a clinical attack.

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STUDIES IN LOUPING-ILL

IV.—PASSIVE IMMUNITY

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INTRODUCTION

In the course of a general investigation, experiments were undertaken to determine the prophylactic value of louping-ill antiserum with a view to diminishing or preventing losses from the disease in young lambs. The present paper deals with the following: (1) Laboratory experiments in passive immunisation; (2) antiserum as a prophylactic in field experiments; (3) studies in maternal transmission of immunity.

In a previous article (Wilson, 1946) an account was given of three methods adopted to determine the louping-ill virus-neutralising antibody values of sera. In the present series the first method, *i.e.*, that in which mixtures of serum and virus were injected intracerebrally in mice, was employed in preference to the other two. The investigation has entailed the examination of many samples of sera. In most titrations the 50 per cent. mortality end-points were estimated according to the method of Reed and Muench (1938). The values of immune sera, recorded in terms of units of antibody per c.c., were calculated on the basis previously adopted where one unit of antibody was taken to represent the amount of serum capable of neutralising 100 mouse infective doses of virus.

LABORATORY EXPERIMENTS IN PASSIVE IMMUNISATION

Duration of Passive Immunity

Since the value of a passive immunity is dependent on its duration, experiments were designed to define precisely the period of time passive immunity to louping-ill would remain efficient.

In the first of the two experiments, six groups, each consisting of three sheep, were in turn passively immunised at weekly intervals using homologous serum containing approximately 4,000 units of antibody per c.c. in doses of 10 c.c. administered subcutaneously. At the end of the 5th week, that is, at the time the sixth group of sheep were immunised, all groups were given a subcutaneous injection of >100,000 M.I.D. (mouse infecting doses) of sheep brain-virus. The interval between administration of serum and virus, therefore, was five weeks in the first group, four weeks in the second

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and so on to the sixth group, which received serum and virus simultaneously in opposite legs. The interpretation of the results was based in the first instance on temperatures which were recorded daily after the injection of virus. Secondly, in the first three groups of sheep a few weeks after the subcutaneous administration of virus, the existence or non-existence of immunity of the central nervous system was determined by the intracerebral injection of virus.

Reference to Chart 1 indicates that, in so far as could be inferred from temperature curves, passive immunity conferred by the injection of 10 c.c. homologous antiserum probably remained durable for a period extending to four weeks but not to five weeks.

As a result of studies in the titration of antiserum by the subcutaneous test in sheep (Wilson, 1946), it could be inferred that if passive immunity were efficient for a period of four weeks there would exist a louping-ill susceptible central nervous system in sheep 82, 83 and 84. This was so in some degree only, for all three animals survived the intracerebral injection of 10^6 M.I.D. of virus; two (82 and 84), however, exhibited on the fourth and fifth days pronounced louping-ill symptoms, including ataxia and much mobility of the neck resulting in marked "dangling movements" of the head when attempts were made to walk or turn. Sheep 83 showed similar symptoms in milder form. In distinction and, as was anticipated, Group I sheep (79, 80 and 81) showed no symptoms. With the exception of one animal (85), which suffered and died from intercurrent affection too early to decide whether it was susceptible or not, Group 3 sheep (85, 86 and 87) developed louping-ill and succumbed as a result of intracerebral injection of virus.

It was concluded that there was a high level of passive immunity for three weeks and that a considerable degree of immunity could persist for approximately four weeks.

In the second experiment each of three sheep (110, 112 and 113) was given 300 c.c. of hyperimmune serum administered partly subcutaneously and partly intravenously in two fractions on successive days. Sheep 112 received homologous serum, 113 received horse serum, and 110 a mixture of equal parts of sheep and horse serum. On the day following administration of the second fraction of serum and at weekly intervals thereafter, sample bleedings were made for antibody content determinations.

In each of the sheep the titre of antibody resulting from the injection of hyperimmune serum, rose from 0 units to >600 per c.c. of circulating serum and no evidence of appreciable fall could be obtained during the following three weeks. During the fourth, fifth and sixth weeks a rapid decrease occurred. In sheep 110, at the end of the sixth week, the amount of antibody present per c.c. of serum appeared to be no more than 0·68 units, while in sheep 112 and 113 the amounts were 68·1 and 6·8 units respectively.

From this experiment it appears that the duration of passive immunity is influenced, though not proportionately, by the size of

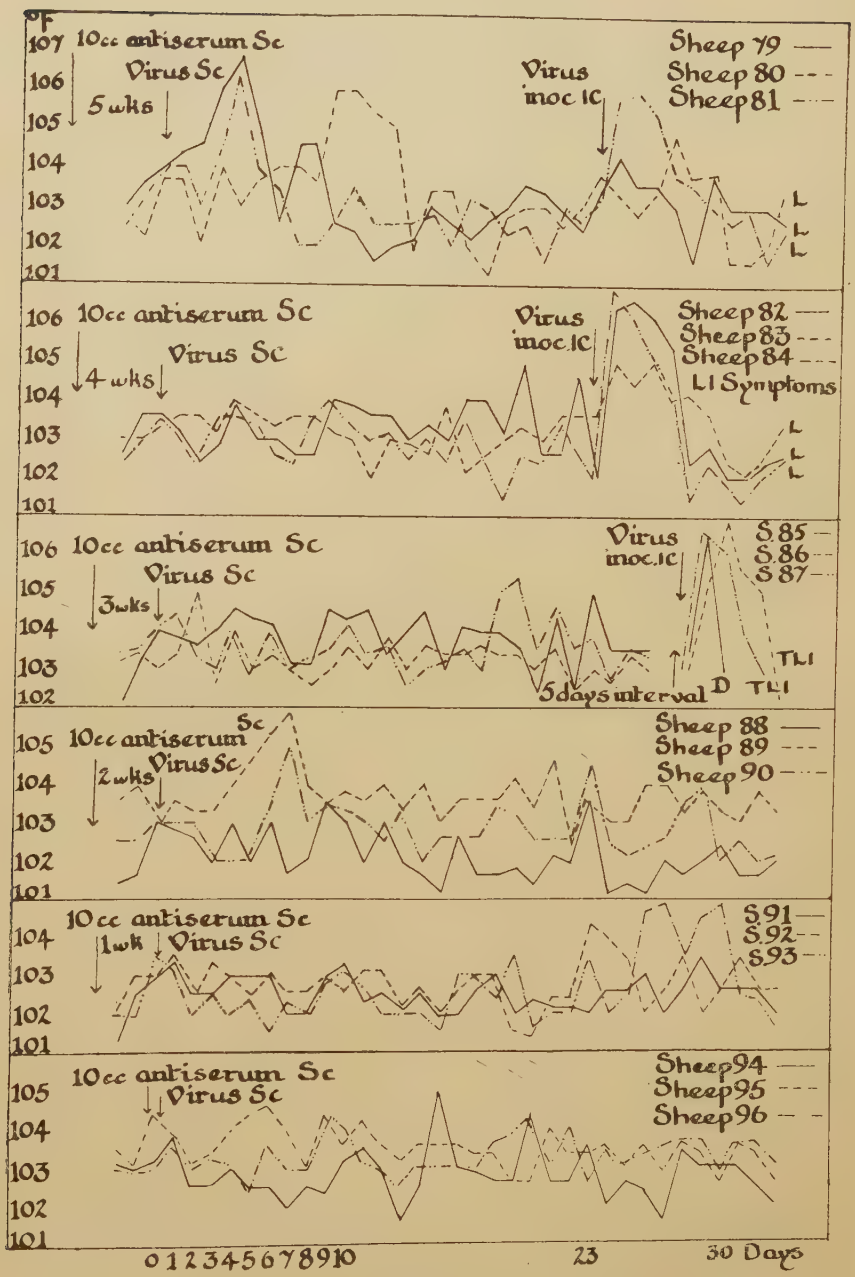

CHART 1

Duration of passive immunity in louping-ill as determined by resistance to experimental infection

Abbreviations. Sc. = Administered subcutaneously.
Ic. = Administered intracerebrally.
T.L.I. = Typical louping-ill.
L. = Lived.

the dose of serum administered. Efficient passive immunity over a period extending to about four weeks apparently could be expected from the prophylactic doses of serum likely to be administered to animals in the field. With doses of serum larger than those customarily employed in prophylaxis, the period might extend somewhat longer.

Effect of High Grade Passive Immunity on Louping-ill Developing from Virus Injected Intracerebrally

Much interest and importance attaches to immunological studies in neurotropic virus diseases, especially that aspect which is concerned with development of immunity in the neural tissues. Although sheep which survive, either natural louping-ill or an infection from the peripheral injection of virus, possess humoral antibodies in amounts varying within fairly definite limits, invariably they are immune to massive doses of virus injected into the brain. Is the establishment of complete immunity merely a question of antigenic response to virus with production of antibody in adequate amount or must some other reason be sought for the unfailing regularity in development of brain immunity in sheep which survive louping-ill infection ? The question arises in connection with passive immunisation as to whether one could ever expect to confer, by subcutaneous administration of antiserum, a complete immunity to louping-ill, that is, an immunity which includes the brain and spinal cord.

In the present investigation, one experiment was performed in which 200 c.c. of hyperimmune serum was administered partly intravenously and partly subcutaneously, in two fractions on successive days, to each of two sheep (567 and 568). On the second day after administration of the second fraction of serum, sample bleedings were made and 30,000 M.I.D. of virus was injected intracerebrally. The amount of serum antibody—almost 400 units per c.c.—which was circulating in each sheep on the day virus was injected, was at least equal to the amount present in many sheep and several times more than in some which had survived infection.

The fact that both experimental sheep developed louping-ill (Chart 2) appears to indicate that pressure of antibody on the visceral side of the blood-brain barrier is not the sole factor which decides susceptibility or non-susceptibility of the nervous tissues of the sheep to louping-ill virus. The lengthened incubation periods of seven and eight days as compared to a normal period of four to five days could possibly have been due to antibody permeating from the vascular system into the brain tissues ; on the other hand, they could conceivably have been due to antibody infiltrating into the nervous tissues at the point where a break was made in the blood-brain barrier at the time of virus injection.

From the point of view of louping-ill prophylaxis, it was concluded from the experiment that under no conditions where anti-

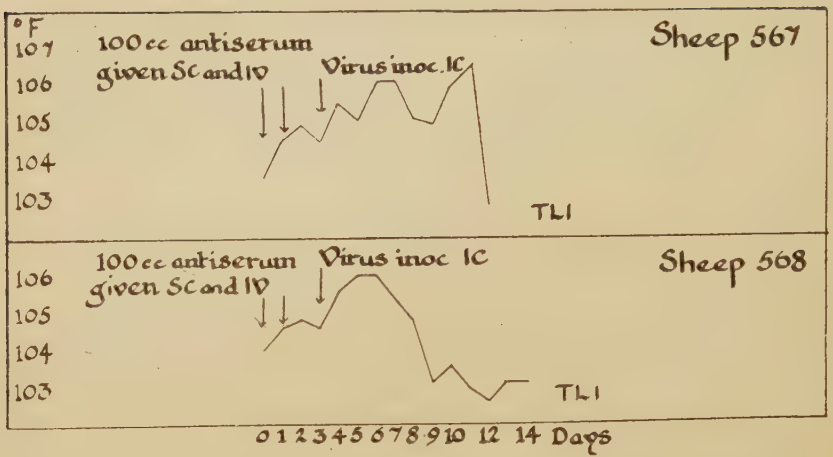

CHART 2

Effect of high grade passive immunity on louping-ill infection developing from intracerebral administration of virus
Abbreviations. IV. = Administered intravenously.
Others as in Chart I.

serum alone was being used could it be expected that the brain and spinal cord tissues would remain otherwise than susceptible.

The Effect of Antiserum on Louping-ill Virus Activity in the Visceral Tissues of Sheep

A developing louping-ill infection induced by the subcutaneous administration of 40,000 M.I.D. of virus was established in sheep 416 to 420 ; each animal in turn then received a single subcutaneous dose of 5 c.c. potent antiserum given at a different time interval after the injection of virus. Thus, sheep 416 received virus and serum simultaneously, 417 received serum 24 hours after the virus, and in the remaining sheep the intervals were 48, 72 and 96 hours respectively.

Reference to Chart 3 shows that in sheep 416, 417 and 418 the temperature curves remained normal, while in 419, which received serum 72 hours after virus, there was some evidence of interruption in what appeared to be the early developing stages of a temperature rise. On interpreting the reactions on the basis of susceptibility or non-susceptibility of the brain tissues as determined by intracerebral injection of virus, it was evident that complete suppression of the primary virus infection by antiserum occurred in sheep 416 (simultaneous) and 417 (24 hours' interval). In 418, which also had a normal temperature, much doubt existed regarding the effect of antiserum since following the test for immunity by the injection of virus into the brain there was a rise in temperature without manifest symptoms of louping-ill.

It therefore appears that 5 c.c. of antiserum administered during

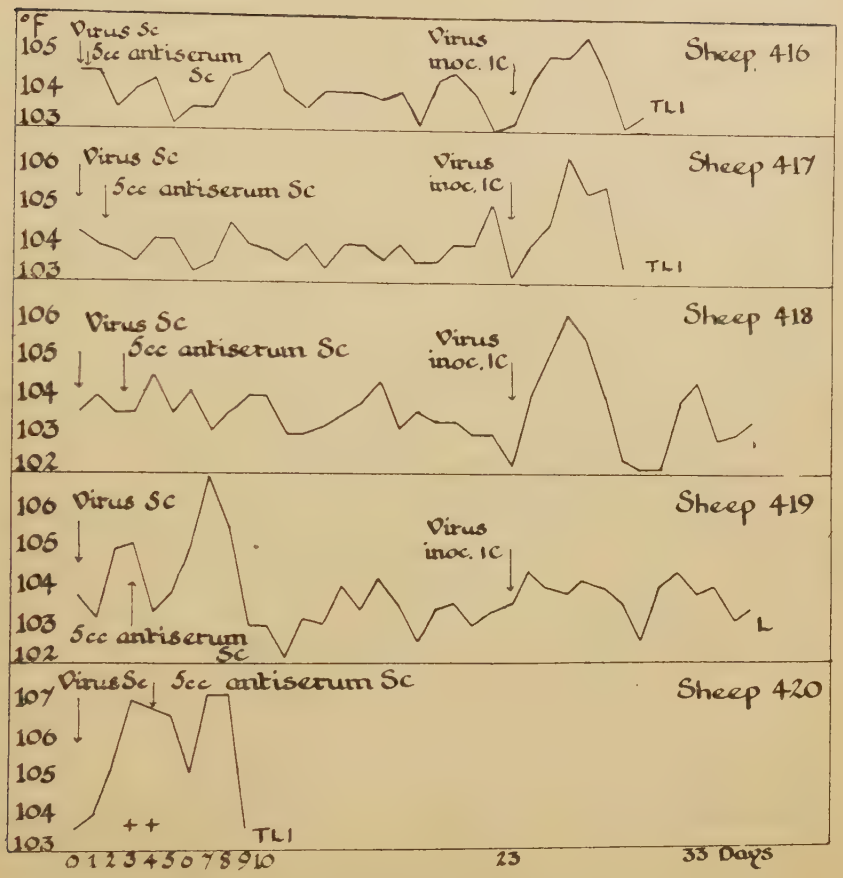

CHART 3

Effect of antiserum on louping-ill infection developing from subcutaneous administration of virus

Abbreviations. + = Virus present in the blood-stream.
Others as in Chart 1.

the very early stages of infection, perhaps only within the first 48 hours, is capable of suppressing the activity of virus in visceral tissues. Once infection progresses to the stage in which a reaction and a temperature rise occur, the administration of a prophylactic dose of antiserum influences only slightly or not at all the further progress of the affection.

LOUPING-ILL ANTISERUM AS A PROPHYLACTIC IN FIELD EXPERIMENTS

Louping-ill antiserum collected from sheep and horses in the course of hyperimmunisation experiments previously described (Wilson, 1946) was tested as a prophylactic against infection transmitted naturally by ticks to lambs in the field. Four tests carried out during three separate seasons were made possible through the

willing co-operation of a number of farmers and stock attendants who themselves performed the serum injections and later furnished their recordings of deaths.

It will be realised that in the absence of *post-mortem* examinations and specific laboratory tests, accurate diagnosis, based only on observed clinical signs, is not always possible and, doubtless, it can be accepted that errors were numerous. This is due not so much to failure on the part of farmers and attendants to recognise symptoms associated with louping-ill, but to the difficulties caused by the frequent occurrence in lambs on some louping-ill farms of nervous symptoms arising from the presence in the stomach or intestines of indigestible materials such as wool, grass ball or sand, or from forms of joint-ill complicated by pyaemic spinal meningitis : moreover, it is not unusual to find lambs dead which, perhaps less than 24 hours before, appeared in normal health.

On each experimental farm approximately 50 per cent. of the lambs were left as untreated controls exposed to the same environment and variety of disease conditions as were serum treated lambs. Although deaths believed due to louping-ill were recorded and are given in the accompanying table, estimates of the prophylactic values of the different antisera employed were formed more particularly on their power to reduce the incidence of death as compared with the incidence in the control lambs which received no serum.

In all experiments 5 c.c. doses of antiserum were administered subcutaneously. In the first and fourth the injections were given to the lambs on the day of birth. In the second and third the farmers were allowed some latitude with regard to time of injection and were permitted to decide for themselves from their previous experiences the most suitable time to administer serum having regard to the period of expected high incidence of cases. In none of the experiments were lambs marked in a fashion which allowed of determining whether the main death incidence occurred during the period following that of expected duration of passive immunity. A summary of the results is presented in Table I.

From laboratory experience one would expect some benefit to be derived from the use of louping-ill antiserum as a means of controlling the activities of the specific virus in the field ; that this was obtained in all four experiments (Table I) is evident from the fact that lower total death-rates occurred in the serum treated than in the numerically comparable untreated groups of lambs. The prophylactic effect of antiserum was most noticeable in Experiment 2, where a total mortality rate of 3·95 per cent. of 5,090 serum treated lambs compared favourably with a total mortality rate of 9·34 per cent. of 6,421 untreated, the difference presumably being due to the lower incidence of louping-ill cases in the former than in the latter group of lambs. Heterologous antiserum as a prophylactic (Experiment 3) was apparently less valuable, although the figures of 6·5 per cent. and 8·32 per cent. death-rates from all causes in the serum treated and control

TABLE I

SUMMARY OF FIELD EXPERIMENTS WITH LOUPING-ILL ANTISERUM AS PROPHYLACTIC

Experiment number and year	*Number of farms*	*Antiserum used*	*Lambs receiving serum*	*Deaths from Louping-ill*	%	*Deaths from all causes*	%	*Number of control lambs*	*Deaths from Louping-ill*	%	*Deaths from all causes*	%
1 (Spring, 1938)	10	Obtained from sheep hyper-immunised with homologous brain-virus	912	11	1·2	41	4·5	907	19	2·09	55	6·06
2 (Spring, 1939)	31	Obtained from sheep hyper-immunised with homologous brain-virus	5,090	63	1·24	201	3·95	6,421	301	4·69	600	9·34
3 (Spring, 1939)	33	Obtained from horse receiving mouse brain-virus as antigen	4,234	73	1·72	275	6·5	4,712	198	4·20	392	8·32
4 (Spring, 1941)	45	Mixture of sheep and horse serum in equal parts obtained from animals hyperimmunised with culture-virus	8,889	328	3·69	1,058	11·90	8,354	442	5·29	1,175	14·07

groups respectively suggest that in the former reduction in the incidence of fatal cases of louping-ill did take place. In Experiment 4, one again presumes that the 2 per cent. difference in incidence of deaths from all causes in the two groups of lambs -11·9 per cent. in serum-treated and 14·07 per cent. in untreated—was due to a decreased number of louping-ill cases in the group receiving antiserum. A feature of this experiment was the high percentage incidence of deaths from all causes occurring in both groups of lambs and it is tentatively suggested, especially since 20 of the 45 farmers participating also took part in the experiments 2 and 3 of 1939, from which data were available, that exposure of the sheep stocks to the hardships of the severe climatic conditions prevailing in spring, 1941, may have been a factor predisposing to the high death-rates recorded.

From these experiments it would seem that the administration of specific antiserum, preferably obtained from sheep, constitutes a method which may be adopted for controlling louping-ill in lambs. In addition to the immediate effect of a noticeable decrease in the number of deaths, and as a result of such decrease, it could be perceived that one other benefit, at present immeasurable, may accrue from the use of louping-ill antiserum. From the epizootiologist's point of view a reduction in the number of cases of louping-ill presumably would result in a reduction in the number of infected ticks which, during their subsequent developmental stages, would be able to transmit virus to other hosts.

STUDIES IN MATERNAL TRANSMISSION OF IMMUNITY

Since the preparation of antisera and also their administration as prophylactics under field conditions entail considerable practical difficulties, studies in maternal transmission of immunity were undertaken. Four experiments were performed and as these furnished evidence that lambs do acquire passive immunity from their actively immune mothers it would appear possible by suitable treatment of adult stocks to ensure in the progeny a satisfactory degree of protection against louping-ill. The conclusions formed are based on the facts obtained in the following experiments involving (1) five lambs born in 1941 from ewes hyperimmunised with live virus ; (2) three lambs born in 1942 from ewes which had been used in Experiment 1, and which, in the subsequent interval of one year, had received either no further hyperimmunising injection (ewes 421 and 409) or only one injection consisting of 5 c.c. formalinised virus vaccine (ewe 696) ; (3) seven lambs born from ewes immunised with vaccine, this forming a method that was safe and applicable in the field ; and (4) experiments in 1944 to determine whether lambs which had sucked the colostrum of immune mothers would develop any degree of active immunity from the subcutaneous administration of moderate doses of active virus.

Lambs Born in 1941 *to Ewes Hyperimmunised with Live Virus*

From each of seven pregnant ewes a sample of serum was procured for storage without preservative about three months before parturition was due. Five of the animals selected for hyperimmunisation against louping-ill were then given one 8 c.c. dose of vaccine (formalinised virus) subcutaneously. Fourteen days later, when immunity was considered established, a course of hyperimmunising injections using as antigen active culture-virus of the second to sixth, but also including 27th and 29th passages, was begun. Over a period of 51 days eleven hyperimmunising injections totalling 400 c.c. of antigen were given to ewes 696, 409 and 702, while over a period of 57 days, 12 injections totalling 500 c.c., were given to ewes 421 and 410, all by subcutaneous route. In each animal parturition occurred within a period of six days after the last dose of virus. The two remaining ewes, 408 and 705, received no treatment and served as experimental controls.

Prior to lambing, the animals were under careful but not constant observation, and on this account the udder of each was covered with cloth in an attempt to prevent the lamb at birth from sucking before a blood sample was obtained. None of the lambs was observed and bled immediately after birth and since, after parturition, the cloth coverings on the udder hung loosely it was not known definitely whether any one of the lambs had been prevented from sucking before the opportunity to do so was given to it.

Blood samples for antibody content determinations were obtained from each ewe and lamb as soon after parturition as was possible, again at three days after parturition, and on two other occasions when the lambs as a group were 65 to 74 days old and 99 to 108 days old.

Reference to Table II, which forms a summary of the results of the titrations of sera, shows that none of the ewes possessed antibody before hyperimmunisation, while in the samples obtained at parturition and at three days after, considerable amounts were present. Of the five samples of lamb serum obtained " at birth," one (lamb 154) was found to contain no antibody ; in the remaining four samples (lambs 159, 160, 161 and 162) the amount present was considerable and corresponded closely per unit of serum to the amount of humoral antibody possessed at parturition by the respective mothers. When three days old, all five lambs possessed antibody, the only notable change having occurred with respect to lamb 154 in that during the three days after birth it acquired a humoral immunity approximately equivalent to that of its mother and of its fellow lambs.

Although the ewes had received the stated number of hyperimmunising injections of living virus, the amount of serum-antibody found at parturition and at three days later was not particularly great in any one, excepting in ewe 409.

With the exception referred to, the amounts were comparable to those found in sheep which survive an attack of experimental

TABLE II

UNITS OF LOUPING-ILL VIRUS-NEUTRALISING-ANTIBODY PRESENT PER C.C. OF SERUM FROM HYPERIMMUNISED EWES AND THEIR LAMBS
(One unit = amount neutralising 100 mouse infective doses of virus)

Ewe and lamb	*Ewe serum obtained before hyperimmunisation*	*Ewe at parturition and lamb at birth*	*Ewe at 3 days after parturition and lamb when 3 days old*	*Ewe at 65 to 74 days after parturition and lamb when 65-74 days old*	*Lamb when 99-108 days old*
Hyperimmune ewe No. 696	0	Not tested	254·8	200	
Lamb No. 154		0	>158·86	35·5	22·4
Hyperimmune ewe No. 409	0	796·2	796·2	632·4	
Lamb No. 159		>158·86	>158·86	?	4·5
Hyperimmune ewe No. 702	0	67·0	67·0	200	
Lamb No. 160		>112·5	>112·5	63·24	4·5
Hyperimmune ewe No. 421	0	178·26	>178·26	200	
Lamb No. 161		67	>112·5	?	7·26
Hyperimmune ewe No. 410	0	178·26	178·26	200	
Lamb No. 162		67·0	67·0	37·68	1·29
Control ewe No. 408		0	Not tested	0	
Control lamb No. 145		0	0	0	0
Control ewe No. 705		0	Not tested	0	
Control lamb No. 147		0	0	0	0

louping-ill induced by a single subcutaneous injection of living virus. Without receiving virus injections during the post-parturient period of the experiment, which extended to three months, each of the hyperimmune ewes retained to a large extent the humoral immunity present at the time of parturition. Apart from the degree of immunity obtained by the lambs from their immune mothers, it was of much interest and importance to note that all five lambs, when 65 to 74 days old, possessed a high immunity, and when 99 to 108 days old at the end of the experiment, humoral antibody, though present in much diminished amount per c.c. of serum, was still detectable.

Lambs Born in 1942 *from Ewes which had been used in Experiment* 1

The sera from three ewes (696, 421 and 409) which had been hyperimmunised with live virus before lambing in 1941, and the sera from their respective lambs (428, 441 and 455) born in 1942 were examined to determine the content of louping-ill virus-neutralising antibody. After an interval of one year during which time no hyperimmunising injection had been administered to ewes 421 and 409 and only one injection, consisting of 5 c.c. formalinised virus vaccine to the remaining animal (696), it was found that high titres of serum-antibody were present in the mothers and that each of the lambs had acquired a humoral immunity about equal in degree to its mother. No antibody was present in the serum of lamb 428, which had not sucked before the blood sample was taken.

Lambs Born from Ewes Immunised with Vaccine

The pregnant ewes were divided into two groups which respectively received four and three injections of vaccine in 5 c.c. doses at 14-day intervals and so timed that the last injection was administered 14 days before parturition was due. Serum samples from each ewe and lamb were obtained for antibody content determinations as in Experiment 1. Summaries of the results of the titrations are presented in Table III.

The immunity response to vaccination in these pregnant ewes (believed to be ten years old) was poor as compared to that generally found in young sheep receiving similar vaccines. Following two vaccinations, only a trace of or no humoral immunity, could be detected, and two of the ewes (388 and 688), after receiving three injections of vaccine, failed to develop detectable antibody in their sera.

However, it was established that antibodies which developed in ewes as a result of vaccination were readily transmitted to the offspring. In general, the higher the degree of humoral immunity which existed in the mother the greater was the quantity of antibody which made its appearance in the serum of the lamb. The small amount of serum-antibody acquired by lamb 436 was probably an exception to this rule but, in this instance, it may be noted that nursing was poor and a supplement of cow's milk was given for

TABLE III

UNITS OF LOUPING-ILL VIRUS-NEUTRALISING-ANTIBODY PRESENT PER C.C. OF SERUM FROM VACCINATED EWES AND THEIR LAMBS
(One unit = amount neutralising 100 mouse infective doses of virus)

Ewes receiving four injections of vaccine

Ewe No.	*Before vaccination*	*14 days after one vaccination*	*14 days after two vaccinations*	*14 days after three vaccinations*	*After four vaccinations*				*Lamb number*	*At birth*	*At 3 days old*	*At 21 days old*	*At 42 days old*
					At parturition	*3 days after parturition*	*21 days after parturition*	*42 days after parturition*					
422	0	0	0·32	3·99	11·25	11·25	20	6·32	426* } twins 427* }	11·25 11·25	6·32 11·25	11·25 20	1·12 0·63
387	0	0	1·0	12·6	20	11·25	20	11·25	429*	0·632	11·25	35·5	6·32
388	0	0	0	0	6·32	6·32	1·12	0·356	432	0	35·56	15·89	0·63

Ewes receiving three injections of vaccine

Ewe No.	*Before vaccination*	*14 days after one vaccination*	*14 days after two vaccinations*	*After three vaccinations*				*Lamb No.*	*At birth*	*At 3 days old*	*At 21 days old*	*At 42 days old*
				At parturition	*3 days after parturition*	*21 days after parturition*	*42 days after parturition*					
416	0	0	0·63	20	20	11·26	0·63	436	0	2·0	0	0
415	0	0	0·63	20	20	35·5	20	439	0	11·25	25·56	20
688	0	0	0	0	0	0	0	440	0	0	0	0

Lamb 436 born from ewe 416 was very poorly nursed and was given cow's milk for several days after birth. This fact probably explains the low serum antibody content as compared to that of its mother.

* In these cases there exists the possibility that the lambs had sucked the ewes before the sample " at birth " was obtained.

several days after birth. The failure of lamb 440 to acquire immunity appears to indicate that the presence of detectable antibody in the mother's serum is necessary before transmission to the offspring can become possible. Serum obtained from each of three lambs (432, 436 and 439) at birth before any one made an attempt at sucking, contained no antibody. It was of interest to note that in this experiment the degree of maternal immunity which was transmitted to lambs, was retained to a large extent for at least six weeks.

Administration Subcutaneously of Living Virus to Young Lambs Born to Immune Mothers

Five pregnant ewes were immunised by the subcutaneous inoculation, at suitable intervals, of 2 doses of louping-ill vaccine (formalinised virus) and three doses of living virus, the last of which was administered about four weeks before parturition was due. Seven lambs were born and when the youngest (twins) were six days old samples of serum were obtained for determinations of the antibody content. At this time also, three of the lambs (755, 759 and 763) were given 73,800 M.I.D. of living virus by the subcutaneous route and thereafter daily temperatures were taken. Judging by these temperatures which remained normal throughout the period of recording, the injection of living virus had had no apparent effect. This did not represent an inherited resistance or non-susceptibility of young lambs since, in other experiments, three lambs of comparable ages but born to non-immune mothers, when given virus by the same route, developed typical thermal responses and virus appeared in their blood streams.

Subsequently, serum was obtained at intervals from all seven lambs to determine whether antibody persisted in the serum of those which had received living virus or whether it disappeared over a period of time as would be expected in the four control lambs (757, 760, 762 and 764) which had not received living virus.

The amount of antibody in the serum of each lamb at the different time intervals is recorded in Table IV, which shows that administration of living virus had had no demonstrable effect for in both groups of lambs a comparable and progressive fall to zero in antibody levels occurred during a period of $>110<138$ days.

Several weeks after antibody had disappeared from their serum, lambs 755, 759 and 763, which previously had been injected with living virus and two (760 and 764) of the four control lambs were given 33,400 M.I.D. living virus by the subcutaneous route. This test indicated a susceptibility to louping-ill, for every lamb developed a thermal response during which period virus was present in the blood stream of 755, 759, 760 and 764, though not demonstrated in the blood of 763; further, two of these lambs (755 and 764), died from louping-ill. It will be noted that 755 had the earlier injection of living virus when very young, but at the time possessed a high grade maternally-transmitted immunity.

TABLE IV

UNITS LOUPING-ILL VIRUS-NEUTRALISING-ANTIBODY PRESENT PER C.C. OF SERUM FROM IMMUNE LAMBS

(Experiment 4)

(One unit = Amount neutralising 100 M.I.D. of virus)

	Units present at time in days after that on which virus was administered					
Lamb number	0 *days*	35 *days*	63 *days*	88 *days*	110 *days*	138 *days*
755*	78·94	82·69	54·67	2	0·672	0·399
757	221·6	91·9	41·89	7·89	0·399	0
759* (Twins)	268·72	113·91	31·13	20	4·74	0
760 (Twins)	163·1	73·2	31·13	10·02	0·738	0
762	126·2	41·62	54·67	2	0·425	0
763* (Twins)	105·27	84·23	19·24	4·23	4·23	0
764 (Twins)	224·8	105·27	54·67	42·26	8·43	0·423

* Lambs which received living virus by subcutaneous route.

From the experiment it was considered that lambs, born in the field to immune mothers and while still young, would be unlikely to develop any degree of active immunity from virus transmitted naturally by tick bite.

DISCUSSION

By the method of test adopted—resistance to experimental infection—passive immunity of the grade conferred to sheep by the administration of prophylactic doses of homologous antiserum would appear to be durable and efficient for approximately four weeks. Beyond this period it is anticipated that there exists little or no residual immunity, although it should be noted that the test was not performed in a manner which took into consideration the possibility of a residual degree of immunity which would not give protection against 100,000 M.I.D. of virus. Since lambs would derive maximum benefit only from a prophylactic method that would ensure an immunity lasting for a period of no less than six weeks, it is evident that antiserum must be regarded as limited in its value. Nevertheless, in controlled field experiments involving several thousand lambs, it was shown that homologous antiserum of high antibody content is capable of effecting an appreciable reduction in the death incidence from louping-ill. In Experiment 1, homologous antiserum, and in Experiment 4, where a mixture of homologous and heterologous antisera were administered at birth, reduction in the deaths from louping-ill was not so marked as in Experiment 2, in which serum was administered at a time which coincided with that of the expected outbreak of the disease, and was thus likely to be of most service. Comparison of the deaths in Experiments 2 and 3, which were run during the same season and thus possibly under the same conditions

with respect to prevalence of louping-ill, shows that homologous antiserum appears to be more effective than heterologous antiserum.

Passively conferred immunity of markedly high grade fails to protect the central nervous system against virus introduced intracerebrally ; it therefore appears that, following the administration of antiserum as a prophylactic under field conditions, immunity should be regarded as being entirely confined to the visceral tissues. The difficulty in conferring passive or active immunity which would embrace the brain and cord in addition to visceral tissues is common to most neurotropic virus affections and with certain exceptions resistance or susceptibility of the neural tissues cannot be correlated with the presence of greater or less amounts of antibody in the blood stream ; indeed, it is believed by many that in these affections, *e.g.*, in anterior poliomyelitis, herpes, rabies, equine encephalomyelitis (and louping-ill appears to be no exception), immunity of the central nervous system can be effected only with living virus, and apparently only when it has succeeded in invading or at least reaching the tissues of that system. There is, however, an interesting exception to this rule. Howitt (1932) and Cox and Olitsky (1936) have noticed that in the experimental infection of the guinea-pig with equine encephalomyelitis virus, antiserum administered by the visceral route or antibody arising from inactive (formalinised) virus, can enhance the resistance of the central nervous tissues to the intracerebral inoculation of even large doses of the infective agent.

From the experiments in which lambs were found to acquire humoral immunity from their actively immune mothers, it may be inferred that there exist in the method possibilities denied to any other that may be adopted for the control of louping-ill in lambs. With one exception already commented on, each of the lambs acquired a grade of humoral immunity approximately equal in degree per c.c. of serum to that of its actively immunised mother and if this fact should prove to be an unfailing feature of maternally transmitted immunity it would certainly be possible to confer to very large populations of lambs an immunity of a grade higher than could be easily possible by antiserum. The more lasting nature of maternally transmitted immunity as compared with that conferred by antiserum is possibly of very great practical importance since it may be inferred that the former would cover the whole period of seasonal activities of ticks during which time lambs are subject to infestation and therefore to virus infection. Whether the three-month period of immunity conferred to lambs in the first and fourth experiments was the result of an initial high antibody titre or of repeated absorptions of antibody from colostrum or milk over a period of time is not clear and still remains to be determined. Lambs in possession of maternally transmitted immunity do not appear to develop any active immunity from moderate size doses of living virus ; by inference it is considered that lambs born to immune mothers in the field are likely to remain merely passively immune though bitten by ticks carrying the active agent.

The production of a grade of immunity in adult stocks in which antibody can readily be detected would appear essential before satisfactory maternal transmission can become possible. This grade of immunity resulting from vaccination would be an asset that, it is believed at present, a large proportion of the adult stocks do not enjoy.

SUMMARY

In louping-ill, the duration of passive immunity conferred by antiserum is dependent in some measure on the amount employed ; with small (10 c.c.) doses the period is about four weeks and, with large doses (300 c.c.), may extend to at least six weeks.

Following the administration of small (prophylactic) doses of antiserum immunity may be regarded as confined wholly to visceral tissues. The immunity of the central nervous system to louping-ill virus is not due solely to pressure of antibody on the visceral side of the blood-brain barrier.

Small (5 c.c.) doses of louping-ill antiserum administered at any time up to about 48 hours after the establishment of infection arrested the virus in visceral tissues. Similar quantities appeared incapable of any effect once infection had progressed to the stage in which a rise in temperature and a systemic reaction had occurred.

The immunity from homologous louping-ill antiserum is more lasting than that resulting from heterologous antiserum. Homologous antiserum appeared more valuable than heterologous antiserum when employed for controlling the seasonal outbreak of the natural disease in lambs.

Lambs born of louping-ill immune ewes acquire a humoral immunity with a tendency for antibody levels to correspond or even to become almost identical with those present in the mothers. Antibody in its entirety is transmitted from mother to lamb by the colostrum or milk. Maternally transmitted immunity may endure for a period of three months. Active immunity does not develop from living virus administered to lambs in possession of such an immunity.

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ATTEMPTED ACTIVE IMMUNISATION OF CATTLE AGAINST *BR. ABORTUS* INFECTION WITH AN ANTIGENIC FRACTION

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INTRODUCTION

In a previous paper, Paterson *et al.* (1947) have described the physico-chemical properties of an antigenic fraction which could be prepared by a variety of methods from a strain of *Br. abortus* (McEwen, 544). There was evidence that this antigen produced an active immunity in guinea-pigs. The degree of resistance engendered was not constantly of a high order, but it could be compared with that produced by a vaccine composed of living avirulent *Br. abortus* organisms (American strain 19). An attempt was made to determine whether this antigen would stimulate an active immunity in cattle, and a group of ten Red Devon heifers which had not been vaccinated against *Br. abortus* infection and which were free from agglutinins, was selected for this purpose.

PREPARATION OF THE ANTIGEN

Several batches of antigen, which had been prepared from dense suspensions of the CO_2-sensitive *Br. abortus* (McEwen, 544) either by digestion with trypsin for 3 to 4 days at 40° C., or by autoclaving in neutral suspension for 15 minutes at 120° C., were mixed together. The fraction which did not sediment on the ultracentrifuge in half an hour at 10,000 r.p.m. but did sediment at 30,000 r.p.m., was used as the antigen for the inoculation of the cattle.

THE INOCULATION OF THE ANTIGEN

Each heifer was inoculated intradermally with 90 mg. of antigen in 2 ml. of fluid. This inoculum was for convenience divided into four equal parts and inoculated on the neck into four sites, situated at the corners of a square of 4 inches side. Within three hours all exhibited a pronounced general systemic reaction marked by severe shivering, high temperature (103·4° to 105·6° F.) and, in several, drooling of saliva. Food was refused for 48 hours and there was a marked loss of condition, the appetites taking 14 days to return fully. Significant swellings developed at the sites of inoculation which, by the seventh day, were hot, painful, tumefied masses approximately 4 inches in diameter. In some cases the separate swellings tended to run together. In all cases the prescapular gland on the inoculated side was prominent and easily palpable. By the 14th day there was a substantial reduction in the hardness and size of all the swellings, most of which exhibited a small necrotic area

with a cornified centre between the 21st and 28th days. Thereafter they subsided slowly, but at most sites a hard knob about the size of a walnut remained permanently.

One hundred and seventy-one days after the first inoculation, each heifer was given 22·5 mg. of antigen in 0·5 ml. fluid intradermally on the opposite side of the neck. The general and local reactions were much less pronounced, although there was some inappetence for a week or so. The local reactions reached their maxima within 24 hours and then subsided slowly over a period of three weeks, leaving no permanent mark.

There was a marked agglutinin response following the first dose of antigen. Fifteen days after inoculation the titres averaged 1 : 5,000, but subsequently they fell away and by the 98th day were at the 1 : 40 to 1 : 80 level. At the last test before reinoculation (168 days) the titres were 1 : 20 to 1 : 40. Reinoculation caused a rise in the titre to between 1 : 640 to 1 : 1,280 14 and 28 days later. The details of the agglutination tests are set out in Table I.

Mating of the heifers by artificial insemination was commenced on 6.2.45, approximately six weeks after the primary vaccination. By 17.4.45, eight heifers had been successfully mated : the two remaining heifers (Nos. II and VI) failed to hold to three services and therefore were discarded from the experiment.

EXPOSURE OF VACCINATED AND CONTROL HEIFERS TO TEST INFECTION

On 20.7.45, approximately six weeks after the inoculation of the second dose of antigen, and when the vaccinated heifers were between three and five months pregnant, each was exposed to infection with virulent, CO_2-sensitive *Br. abortus* (McEwen, 544) by instilling 0·1 ml. of a hundredfold dilution of a Brown 10 suspension on to the conjunctiva of the eye. At the same time four similar brucella-negative heifers at a comparable state of pregnancy were infected by the same route. These latter heifers were at the Ministry of Agriculture's laboratory at Weybridge, and were controls for two independent vaccination experiments which were being carried out concurrently at Cambridge and Weybridge. The suspension was prepared at Weybridge in the morning and part was despatched to Cambridge by messenger. Both vaccinated and control groups were inoculated at a prearranged time in the afternoon. A viable count of the suspension used at Cambridge showed that each vaccinated heifer received approximately $14{\cdot}6 \times 10^6$. The virulence of the infecting dose for guinea-pigs was titrated at Cambridge. Four groups each of five animals were inoculated with an estimated dose of 40,600, 4,060, 406 and 41 living brucella organism respectively ; they were killed 42 days after inoculation. With the three larger doses all the guinea-pigs became infected ; with the smallest dose three out of the five were free from infection. This titration indicates that the suspension used was fully virulent for guinea-pigs.

TABLE I

SUMMARY OF AGGLUTINATION TITRES, HISTORY OF PREGNANCY AND BACTERIOLOGICAL EXAMINATIONS

Heifer No.	Agglutination titres: Days after inoculation of — 1st dose antigen 90 mg.											2nd dose antigen 22·5 mg.				Days after exposure to $14{\cdot}6 \times 10^6$ infection viable Br. abortus, strain 544									History of pregnancy			Bacteriological examination (cultures and guinea-pigs)			
	0	8	15	21	28	42	56	77	98	119	147	168	7	21	35	14	28	42	56	77	98	119	140	161	Days between service and infection	Nature of calving	Duration of pregnancy in days	Titre of blood at calving	Foetal membranes	Foetus	Milk samples: Colostrum	Milk samples: 2 wks.	Milk samples: 4 wks.
I	0		10	10	10	7	5	4	4	3	2	2	8	8	6	5	6	6	6	5	4	8			138	Normal	272	8	...	...	—	...	...
II	0	9	9	8	8	6	4	3	3	2	2	2	7	8	6	Non-pregnant—discarded																	
III	0	10	9	9	9	7	5	4	4	4	3	3	9	8	6	9	6	6	9														
IV	0	10	9	9	7	7	5	4	4	3	3	3	9	8	4	5	5	5	5	6	7				164	Aborted	232	7	+	+	+	...	...
																									151	Premature	261	6	+	...	—	+	...
VI	0	8	12	10	9	7	5	4	4	3	3	3	9	7	6	Non-pregnant—discarded																	
VII	0	8	9	8	8	7	5	4	4	3	2	3	7	7	5	3	3	4	4	6	2	3	3	4	131	Normal* (dystokia)	287	4	—	—	—	—	—
IX	0	10	10	9	8	6	4	2	3	2	2	3	7	6	6	3	4	5							163	Aborted	203	4	+	+	...	...	...
X	0	9	10	8	7	5	4	2	3	3	3	2	6	6	5	3	4	4	4	4	5	8			95	Aborted	246	8	+	+	—	+	...
XIV	0	9	9	8	7	6	4	2	2	2	2	2	7	6	6	2	3	3	2	2	2	3	3	6	154	Normal	280	6	+	...	—	—	—
XVI	0	10	9	8	8	6	6	4	4	4	3	3	7	7	4	4	5	4	3	4	4	3	4	4	134	Normal	283	4	—	...	—	—	—

Interpretation of symbols :

Agglutination tests serum dilutions

1 = 1 : 10 2 = 1 : 20 3 = 1 : 40 4 = 1 : 80 5 = 1 : 160 6 = 1 : 320 7 = 1 : 640 8 = 1 : 1280 9 = 1 : 2560
10 = 1 : 5120 11 = 1 : 10240 12 = 1 : 20480

* Calf dead when delivered + = *Br. abortus* isolated ... = No examination made — = *Br. abortus* not isolated.

RESULTS

The techniques employed for the bacteriological examination of the heifers at the time of calving have previously been described (Edwards *et al.*, 1945 ; Paterson, 1947).

Control Heifers.—Each of the control heifers developed a positive blood agglutinin titre to *Br. abortus*, aborted a dead calf and virulent CO_2-sensitive *Br. abortus* was isolated from the membranes and from the foetus in each case.

Vaccinated Heifers.—Three of the eight heifers aborted dead calves, virulent CO_2-sensitive *Br. abortus* being recovered in each instance from the foetal membranes and the foetus. One heifer produced a premature calf which lived, but typical cultures of *Br. abortus* were recovered from the foetal membranes. Four heifers gave birth to calves at the normal time. From two of these heifers *Br. abortus* was isolated from the foetal membranes. The other two heifers apparently resisted infection and brucella cultures were not obtained from the membranes or the colostrum or subsequently from the milk. One of these two negative heifers had a difficult calving and the calf was dead when delivered. Bacteriological examination of this calf was also negative. The details regarding service, pregnancy and calving are set out in Table I.

DISCUSSION

It is doubtful if the inoculation of the antigen had any appreciable immunising effect on the group of heifers studied. It is not unusual for one out of ten normal pregnant heifers to resist infection when approximately 15×10^6 virulent brucella organisms are instilled on to the conjunctiva. The fact that two heifers treated with antigen resisted infection does not carry any great significance.

The rise and fall of circulating agglutinins following the initial dose of 90 mg. of antigen was very similar to that invoked by 60×10^9 S 19 organisms (Edwards *et al.*, 1946). The second inoculation of 22·5 mg. of antigen six weeks before the test dose gave a marked boost to the blood titre so that at the time of infection the titres were much higher than in the comparable S 19 animals described by Edwards *et al.* (1946). This is further evidence that the presence of circulating antibodies does not mean that the resistance of an animal is high. There is, however, the possibility that in our animals the initial dose was excessive and the severe reaction which followed might have undermined the mechanism of resistance in some way. Better results might be obtained if the initial dose were lowered and the boosting dose increased. The evidence at present available indicates that the fraction is not a complete immunising antigen and that further study is necessary.

SUMMARY

The inoculation of 90·0 mg. of a brucella antigen before mating followed by a further 22·5 mg. during pregnancy, may have slightly

increased the resistance of a group of eight heifers to subsequent experimental exposure to infection with virulent *Br. abortus*. This increase in resistance was manifested by two heifers which completely resisted infection, two others which, although infected, gave birth to living calves at the normal time and a fifth heifer, also proved to be infected, which had a premature living calf. The degree of protection afforded by two spaced doses of the antigen to cattle is much lower than that which results from a single inoculation of 60×10^9 strain 19 organisms, as described by Edwards *et al.* (1946).

The initial dose of 90·0 mg. provoked a marked agglutinin response, marked local reactions and severe systemic disturbances. The local and general reactions following reinoculation with 22·5 mg. were not so severe.

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THE CONCENTRATION OF PENICILLIN IN THE MILK AFTER INTRAMMARY INFUSION

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INTRODUCTION

There have been a number of reports on the concentration of penicillin in the milk of cows at various time intervals after injection via the teat duct. In most instances the penicillin has been dissolved in distilled water but in some saline and oily suspensions have been used. In all the work reported the number of animals in any group has been small (in several cases only one animal), and since the assay has usually been on fore milk samples, great variations in unitage have been reported. (Weirether *et al.*, 1945; Murnane, 1945, 1946; Bryan *et al.*, 1945; Schalm and Casselberry, 1946; Stevenson, 1946; Thorp *et al.*, 1947; Kelly and Bell, 1947.)

The combined evidence, however, shows that there is a relatively high but very variable level of penicillin in the milk 12 hours after injection of a dose of approximately 20,000 to 30,000 units and that there is often some at 24 hours. Murnane (1945) and Schalm and Casselberry (1946) state that the level is inversely related to the milk yield while Weirether *et al* (1945) state that there is no difference in the levels between " pathologic " and normal glands unless *Bact. coli* is present.

Since it appeared that 20,000 to 25,000 units was approximately the therapeutic dose for this disease and that this was becoming increasingly used (A.R.C. Rep., 1946; Murnane, 1946; Moon, 1946; Watts, 1947), it was decided to ascertain the concentration of penicillin in the milk of a large number of cows after all their quarters had been injected with this dose. It was hoped also to determine some of the factors which affect this concentration.

Thus the work to be described relates solely to the effects of the injection of 20,000 units of penicillin in 50 ml. of distilled water, except in one series in which it was reduced to 1 ml.

TECHNIQUE

Every animal was milked out and injected in all four quarters irrespective of whether they were infected or not. Fore milk samples consisted of 15 ml. of the first milk drawn, bulk milk samples of 20 ml. of milk from the complete yield of the cow and strippings of 15 to 20 ml. of the last milk obtainable after removal of the machine. Samples from the morning milking were examined that day and evening samples the following morning. Two methods of examination

were used in duplicate—the cup method of Florey *et al.* (1941), using *Staphylococcus aureus* as the test organism, and dilution in litmus milk tubes as described by Watts and McLeod (1946). The milk for these latter tests was prepared from dried skim milk, each batch being tested for its suitability before use. Fresh skim milk varies considerably and is often not suitable for this purpose. *Streptococcus agalactiae* habituated to the particular batch of milk being used was employed. The unitage of the particular sample being tested was derived from the mean of the readings of the two methods. All solutions used for injection were assayed at the time of injection.

Mastitis was considered to be present in a quarter if the fore milk yielded *Str. agalactiae* on each of three successive tests taken within 14 days of the injection of penicillin. It was considered absent if no sign of pathogenic bacteria was found in these examinations. Doubtful quarters have not been included.

RESULTS

The Average Concentration in the Milk and the Variation according to Yield

Since Murnane (1945) and Schalm and Casselberry (1946) have already reported that the concentration of penicillin depends on the amount of milk present, the results were first analysed on this basis. Table I gives the data for 119 animals, the yield given being that of the morning milking immediately following injection. The assay

TABLE I

THE RECOVERY OF PENICILLIN RELATED TO THE MILK YIELD
(Cow samples 12 hours after injection)

Yield of milk (in lb. per milking)	*No. of cows*	*Average No. of units of penicillin per ml.*	*Average total recovery in units*
<5	10	3·2	3,800
5·1–10	28	1·5	5,500
10·1–15	36	2·5	15,100
15·1–20	37	2·8	24,600
>20	8	5·2	55,100
Total	119	2·6	17,600

was made on the complete yield of the cow at the milking 12 hours after injection. The average results given in the last line of the table are within the range reported by other workers, but when grouped according to the milk yield, as in the first five lines of the table, they do not support the contention that the concentration varies inversely with the yield. Schalm and Casselberry (1946) used fore milk samples and their results are therefore not strictly comparable. Murnane (1945) apparently used bulk samples: he states that his figures are the average for "several" cows injected with

15,000 units in 100 ml. and gives a " full milk " unitage of 0·6 ml. and a " moderate volume of milk " unitage of 4·0 ml. Thus, the cows in moderate milk show seven times the unitage of those in full milk a result which can hardly be explained by the dilution factor. Our average unitage in each yield group did not vary so greatly as Murnane's and what variation did occur was of no statistical importance: indeed, the lowest average concentration was in a group of low yielding cows. Since the concentration did not vary greatly from group to group the figures for the total recovery of penicillin given in the last column show an increase in the total recovery of penicillin directly related to the milk yield. These are for all the results together, but we had noted repeatedly that the unitage recovered varied according to the timing of injections. Thus on some days all the samples showed a relatively high unitage to both methods of assay done independently, while in others the overall figure was lower. To exclude this factor, we analysed the figures for one farm of 31 animals where all the quarters of all the cows were injected simultaneously and the unitage in the bulk milk of each cow was ascertained at 12 and 24 hours and in the fore milk at 24 hours. The results (Table II) show that there was some difference between the unitage recovered from cows yielding less than 5 lb. per milking

TABLE II

MILK YIELD AND AVERAGE UNITAGE IN A SINGLE HERD OF 31 COWS

Yield in lb./milking	*No. of cows*	*No. of quarters*	*Average units of penicillin per ml. in*		
			Bulk milk at 12 hours (cow samples)	*Bulk milk at 24 hours (cow samples)*	*Fore milk at 24 hours (quarter samples)*
1·0–5	5	20	4·8	0·96	1·45
5·1–11	16	64	1·4	0·11	0·36
11·1–17	12	48	2·0	0·26	0·43

and those yielding more, but that no significant difference was shown between the groups giving 5 to 11 lb. and that over 11 lb. In our experiments, therefore, the variation in the unitage of penicillin recovered from experiment to experiment and from cow to cow within any one group was too great to show whether an inverse relationship between the unitage of penicillin and the milk yield occurred in cows giving a reasonable yield of milk.

The Variation in the Concentration according to the Chloride Content, the Stage of Lactation and the Age of the Cow

The chloride content of the milk either before or after injection could not be shown to affect the results. In a series of 186 examinations the average level was the same whatever the unitage of penicillin recovered. This held true both for the percentages of chloride

before injection and after injection. In bulk samples, there was a average increase of 0·02 per cent. of chloride 24 hours after injection.

Similarly, neither the stage of lactation nor the age of the cow, the figures for which are set out in Tables III and IV, affected the results. Since, as shown later in this paper, mastitis may influence the concentration of penicillin, the animals have been subdivided into mastitis positive and mastitis negative groups: the results, however, are still unaffected. The small increase shown in the mastitis negative group over seven years old is not significant.

TABLE III

THE RECOVERY OF PENICILLIN RELATED TO THE STAGE OF LACTATION
(Cow samples 12 hours after injection)

Stage of lactation		*No. of cows*	*Average unitage*	*Average total recovered*
< 50 days	Mastitis +	7	2·5	15,300
	„ —	7	2·9	15,900
	Total	14	2·7	15,700
51–100 days	Mastitis +	9	2·0	9,200
	„ —	14	3·2	27,800
	Total	23	2·7	20,400
101–150 days	Mastitis +	17	1·4	7,200
	„ —	20	3·1	23,600
	Total	37	2·3	16,100
151–200 days	Mastitis +	7	1·5	7,400
	„ —	11	2·6	18,400
	Total	18	2·2	15,300
> 200 days	Mastitis +	15	2·9	11,800
	„ —	7	4·0	25,300
	Total	22	3·2	16,100

TABLE IV

THE RECOVERY OF PENICILLIN RELATED TO THE AGE OF THE COW
(Cow samples 12 hours after injection)

Age	*Udder condition*	*No. of cows*	*Average unitage*	*Average total*
2–3	Mastitis +	3	1·8	11,000
	„ —	29	3·0	21,000
	Total	32	2·9	19,900
4–6	Mastitis +	13	1·6	10,300
	„ —	19	2·8	23,000
	Total	32	2·3	17,800
7 and over	Mastitis +	33	2·0	8,900
	„ —	9	4·5	31,600
	Total	42	2·6	13,800

The Variation in the Concentration according to the Presence or Absence of Mastitis

The criteria for alloting a quarter to one or other of these two groups have been described in the section on Technique. A total of 121 cows was injected, 63 being clear and 58 infected. The average unitage per ml. in the milk of the non-infected cows 12 hours after injection was 3·2, while in the infected cows it was 2·2. The standard error of these two means is 0·35 and 0·21 respectively and p is less than 0·01. The results are, therefore, significantly different between the two classes and it would appear that a greater concentration of penicillin occurs in normal than in infected quarters.

A similar assessment was made of the concentration of penicillin in the bulk milk of each cow 24 hours after injection. The average unitage was identical for both groups, *viz.*, 0·16 where mastitis was present and 0·15 where it was absent. In each case exactly half the cows showed no assessable value of penicillin in the bulk milk 24 hours after injection, but fore milk samples which were taken in some of the cows usually showed a low level of penicillin. The figures are given in Table V, where the non-infected quarters have been divided into two groups : those in cows one or more of whose other quarters

TABLE V

PENICILLIN UNITAGE IN FORE MILK SAMPLES 24 HOURS AFTER INJECTION

Total quarters in mastitis-infected cows		*Infected quarters*		*Non-infected quarters in*			
				Infected cows		*Non-infected cows*	
Number	*Average unitage*	*Number*	*Average unitage*	*Number*	*Average unitage*	*Number*	*Average unitage*
66	0·34	41	0·27	25	0·46	56	1·1

showed infection and those in totally free cows. When the results for non-infected quarters in infected cows are compared with those for the infected quarters the following figures are obtained : mean unitage in infected quarters 0·27 $\pm$ 0·04, and in non-infected quarters 0·46 $\pm$ 0·08 (p greater than 0·05). Thus there is no statistically significant difference between the concentration of penicillin in the milk from infected or non-infected quarters of the same cow. When however, the figures for the non-infected quarters in non-infected cows—mean 1·09 $\pm$ 0·16—are compared with those for similarly non-infected quarters but in infected cows—mean 0·46 $\pm$ 0·08—the difference is significant, p being less than 0·01. The concentration of penicillin in the fore milk of any quarter 24 hours after injection is therefore affected by the presence or absence of mastitis in the cow. If she is completely free there will be a relatively high level of penicillin. If she is infected the concentration in any quarter, whether or not itself actually showing *Str. agalactiae*, will be significantly

less than if she were free, while it is probable, though not statistically significant on the figures available, that the concentration in the free quarters will be higher than in the infected quarters of the same cow.

The Variation in Concentration according to the Volume of Diluent

A comparison was made of the concentration of penicillin in the bulk milk 12 and 24 hours after injection of 20,000 units in 50 ml. and 1 ml. of diluent: in addition, fore milk samples of individual quarters were tested at 24 hours and the chloride content and cell counts recorded. There were 23 cows in the 50 ml. group and 25 in the 1 ml. group. In so far as type of farm, degree of infection and stage of lactation were concerned, the groups were composed of equal numbers of cows. The average concentration in the first group 12 hours after injection was 1·35 units with a standard error of 0·3, and of the second 2·28 $\pm$ 0·33 with p less than 0·05, indicating that the differences are very probably significant. In other words, if the penicillin is injected in the smaller quantity of diluent there is a greater concentration in the milk at 12 hours than if it is injected in a larger quantity while at 24 hours the difference between the two was even greater. The average concentration in the milk of the 23 cows injected with 50 ml. was 0·1 units, but there was no assessable penicillin in 13 of them. The average in the 25 cows injected with 1 ml. was 0·3 units with only three showing no assessable penicillin. There was no significant difference in the cell and chloride contents of the two groups.

This slightly higher concentration of penicillin, when injected in small amounts of diluent, agrees with the charts given by Thorp *et al.* (1947) for four individual cows, each of which had a different dose of penicillin. The fore milk samples taken six hours after injection showed in each case a higher level of penicillin when the diluent (saline) had a volume of 20 ml. than when the volume of diluent was greater.

Thus there is strong evidence that if penicillin is injected in small quantities of diluent the concentration is maintained at a higher level than if a greater quantity of diluent is used.

The Variation in Concentration on Repeated Injection

During the course of the above work it was noted that great variations in the unitage of penicillin occurred according to the group of experiments. Thus, on any one day the unitage recovered might be higher or lower than the average, but whichever it was it would be uniform in all the animals. It was rare for very low and very high recoveries to be made on the same day. This was unlikely to be due to variations in the dosage of penicillin as the injection was assayed simultaneously with the samples. Neither could it be the method of assay since this was carried out in duplicate, using two methods adequately controlled by solutions of penicillin of known strength.

It was considered that the three most likely reasons were: (*a*) idiosyncrasy on the part of the animals since usually all the animals on one farm were assayed together; (*b*) the technique of administration; (*c*) allergic response.

To study the first of these possibilities, five animals were chosen in a herd with little mastitis. Three were heifers in their first lactation and two were cows, which had been exposed to infection when younger at a time when there was considerable infection in the herd. All were in early lactation and were injected with 20,000 units in 50 ml. of distilled water every fortnight for five months, the concentration of penicillin being assayed 12 hours and 24 hours after each injection. Samples of the complete milk and of the strippings were taken. The same animals were therefore exposed to repeated injections of penicillin. If the unitage depended mainly on the group characteristics of the cows it would be expected that every test would show approximately equal concentrations of penicillin. The figures given in Table VI show that this was not so but that there were certain variations.

TABLE VI

UNITS OF PENICILLIN IN THE MILK AFTER REPEATED INJECTIONS AT INTERVALS OF APPROXIMATELY 14 DAYS

Injection No.	*Carnation*				*Stella*			
	12 *hours*		24 *hours*		12 *hours*		24 *hours*	
	B	*S*	*B*	*S*	*B*	*S*	*B*	*S*
1	1·4	1·5	0	0	1·4	1·4	0	0
2	4·0	5·0	0·08	0·08	4·0	6·5	0·16	0·4
3	3·9	4·2	0·40	0·60	3·0	3·1	0·1	0·3
4	1·4	2·2	0·0	0·0	1·8	1·2	0	0
5	1·2	1·2	0·08	0·08	0·5	0·5	0	0·1
6	2·5	2·0	0·08	0·12	0·03	0·06	0	0
7	1·2	1·2	0·08	0·16	0·12	0·16	0	0
8	1·2	1·5	0·16	0·16	0·5	0·4	0	0

Injection No.	*Trixie*				*Dorette*				*Gadfly*			
	12 *hours*		24 *hours*		12 *hours*		24 *hours*		12 *hours*		24 *hours*	
	B	*S*	*B*	*S*	*B*	*S*	*B*	*S*	*B*	*S*	*B*	*S*
1	1·3	0·9	0	0	0·5	0·4	0	0	0·7	0·3	0	0
2	3·0	2·5	0·16	0·1	1·2	1·2	0	0	1·2	2·5	0	0
3	2·8	3·6	0·08	0·1	1·9	1·9	0	0	1·5	1·7	0	0
4	1·4	1·3	0	0	0·5	0·4	0	0	0·6	0·6	0	0
5	1·0	1·2	0·08	0·1	0·5	0·5	0	0·08	0·3	0·7	0	0·08
6	1·2	0·6	0	0	0·4	0·6	0	0	0·7	1·2	0	0
7	1·2	1·0	0·08	0·08	0·16	0·24	0	0	0·5	0·5	0	0
8	1·5	1·1	0·08	0·16	0·3	0·4	0	0·08	0·4	0·8	0	0·3

B = Bulk. *S* = Stripping.

Individual Cow Variations.—Carnation and Trixie, two of the three heifers, consistently showed higher yields of penicillin than did the two cows, Dorette and Gadfly. This variation may have been due to age, but this is doubtful if the previous analysis of all the figures is correct. Another possible explanation is that the two older animals had been in a mastitis infected herd and their udders might have been infected and had then recovered. Mastitis, as shown above, may have some effect on the amount of penicillin present and these animals may have suffered some sub-clinical damage to their udders.

Test Variations.—There were bigger variations between individual tests. Thus the first experiment yielded a low average concentration of penicillin at 12 hours and none at 24 hours. The second and third tests both gave high yields of penicillin at 12 hours in all five animals, while the three young animals, even at 24 hours, showed a low concentration of penicillin in the bulk milk. The remaining tests varied between low and high average concentrations.

The variation in concentration noted in the general experiments was noted again in this series despite the use of the same cows. It could not, therefore, be due to any idiosyncrasy on the part of certain groups of cows of any particular breeding.

The second possible factor—the technique of administration—was also investigated. The diluent had been constant throughout the whole series but the batch of penicillin had varied though this variation could not be definitely related to the concentration of penicillin recovered. The two most likely possibilities were, therefore, considered to be the batch of penicillin and the temperature of the injection. To examine the first of these two possibilities, six different batches of penicillin were injected into 12 normal cows in mid lactation, each batch being injected into two cows, *i.e.*, eight quarters. They were prepared by three different firms and varied in purity from 151 O.U. per mg. to 1,260 O.U. per mg. The milk yield was checked and the chloride percentage and cell count ascertained before and after injection. The concentration of penicillin, estimated on both bulk samples and quarter samples at 12 hours and 24 hours after injection, tended to be high but no significant difference could be noted between the batches at the 12th hour. One cow injected with a batch of penicillin of medium purity which had been used in many animals without causing a fall in yield, recorded a drop in milk of 10 lb. and showed the lowest concentration of penicillin. Its companion, however, remained normal.

The estimations at 24 hours and those on separate quarter samples also showed no differences between the batches of penicillin, and there were no obvious variations in the cell counts or chloride concentrations.

The possible effect of the temperature of the injection was then studied since although the samples were at 40° C. when they left the laboratory, various delays could have cooled them on some occasions

though it was not likely to have been very great. To test this effect, eight normal cows were injected with 20,000 units in 50 ml. of distilled water per quarter. The temperature of the injection was 20° C. in four, 40° C. in two, and 4° C. in two. Even the last injection had no greater effect on the milk yield, chloride content or cell count than the injection at 40° C. To confirm that possible reactions were not masked by differences in individual cows, four of the animals were injected on three further occasions at which the temperature of injection was alternated. The results of these experiments, given in Table VII, show that temperature does not affect the concentration of penicillin in the bulk milk at 12 hours or the milk yield of the cows.

TABLE VII

THE TEMPERATURE OF THE INJECTION RELATED TO MILK YIELD AND PENICILLIN CONCENTRATION

Temperature of injection (°C.)	*Cow*	*Yield in lb./day*		*Chloride Percentage*		*Cell count (thousands/ml.)*		*Penicillin concentration units/ml. at 12 hours*
		Pre Injection	*Post*	*Pre Injection*	*Post*	*Pre Injection*	*Post*	
40	Cynthia	18·5	14·0	0·11	0·16	780	18,000	0·50
40	„	17·0	14·0	0·15	0·14	3,300	36,000	1·00
40	Dewdrop	31·5	30·0	0·10	0·10	330	3,000	2·00
40	„	32·5	32·0	0·10	0·10	270	10,000	1·70
40	Echo	26·5	25·5	0·07	0·08	60	400	3·00
40	„	30·5	27·0	0·08	0·08	120	13,000	0·50
40	Glamour	17·0	14·5	0·12	0·10	500	2,800	0·70
40	„	20·5	13·0	0·09	0·10	550	11,000	0·16
20	Cynthia	15·5	15·5	0·11	0·14	300	14,000	3·50
20	„	18·5	15·0	0·12	0·13	600	35,000	2·25
20	Dewdrop	26·9	25·5	0·09	0·09	200	3,000	3·25
20	„	29·0	32·0	0·09	0·10	210	4,000	1·75
20	Echo	29·5	30·0	0·08	0·09	390	2,400	0·30
20	„	32·0	32·0	0·07	0·09	800	4,300	0·30
20	Glamour	19·5	15·5	0·12	0·13	930	800	0·06
20	„	19·0	19·0	0·13	0·12	1,900	5,300	0·16
20	Dora	19·0	25·5	0·12	0·12	2,130	3,670	0·16
20	Misty Morn	19·0	21·0	0·11	0·12	1,000	1,670	0·32
4	Giddy	22·0	22·0	0·10	0·10	270	2,400	0·64
4	Carnation	21·0	21·5	0·10	0·12	1,070	2,130	1·00

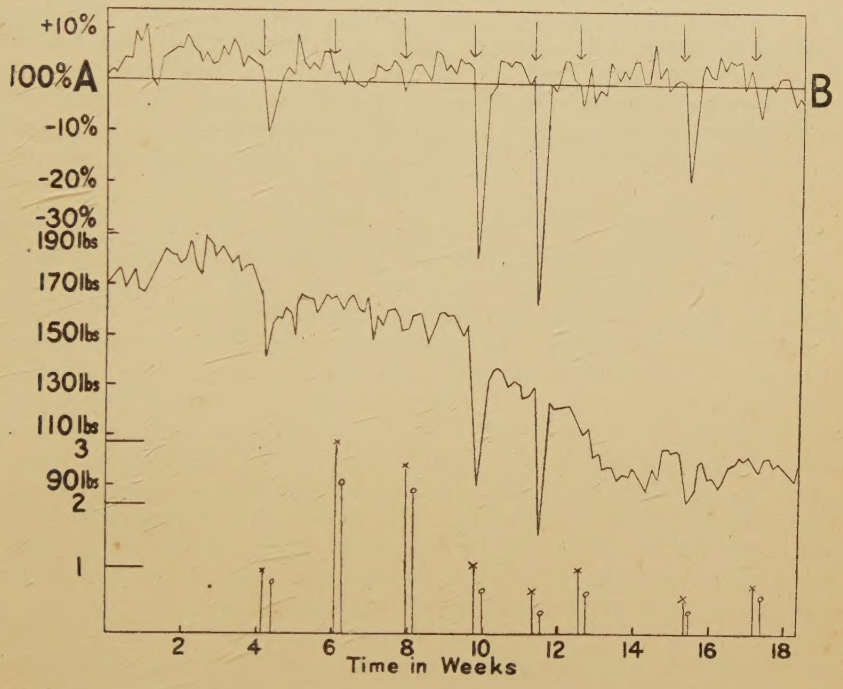
Post injection figures for chloride and cells relate to cow samples 12 hours after injection.

No evidence could, therefore, be found to indicate that the two factors discussed above caused the variations. However, the owners of cows had occasionally reported that the yield had been greatly reduced following injection. In one such case 20 animals all injected at one time had shown such a response. While a little doubt existed as to one or two of them, the vast majority, if not all, had been previously injected at least once. The milk yields of the cows which

had been repeatedly injected in the experiment described above were, therefore, examined to ascertain if they would show any particular response which could be related to the concentration of penicillin and which might have been due to some allergic factor.

It was found that when the concentrations of penicillin were low the milk yield fell considerably after injection. The figures are shown graphically in Fig. 1, where the combined average daily yields of the five injected animals are given and compared with the similarly averaged yields of five controls. The lower curve in the graph gives the actual average daily milk yield of the five experimental cows.

FIG. 1.



The arrows at the top show the dates of injection and the heavy lines at the bottom headed by the letter x, give the actual unitage of penicillin per ml. in the milk 12 hours after injection. The thinner lines, headed by " o," represent the total percentage recovery of penicillin. The figures 1-2-3 on the ordinate of the graph refer to units of penicillin per ml. of milk, the figures in lbs. represent the actual daily milk yield of the experimental animals and the percentage of figures relate this yield to the average of the control group (AB). Higher concentrations of penicillin were recorded during the period of full milk except on those occasions when the yield showed an abrupt fall after injection, in particular the fourth and fifth, when it was accompanied by a corresponding

fall in the concentration of penicillin and not by a rise, as would have been expected had the concentration depended greatly on the degree of dilution by milk. This phenomenon occurred in all five animals and may have been due to an allergic response but could have arisen from the physiological state of the udder since, as shown in the lower curve in the graph, it occurred when the milk yield was beginning to decline. It is not possible to state whether or not this physiological state caused or contributed to the reaction but in any case no permanent effects on the yield resulted. Although the average yield did not attain the previous level, this fall was common throughout the herd as shown by the top graph in which the line AB represents the average daily milk yields of five control cows. Thus AB represents 100 per cent. : where the graph is above this line the experimental animals gave more than the controls and where it is below they gave less. It will be seen that after the abrupt fall they rapidly returned to the normal yield of the herd. The cause of this response to the injection of penicillin is being further investigated particularly with reference to the possibility of an allergic factor.

DISCUSSION AND SUMMARY

In this work the concentration of penicillin in the milk has been shown to be affected by several factors. Although in a series of 130 cows the dosage and volume of diluent have been constant, it has not been possible to confirm the reports that the concentration varies inversely with the volume of milk secreted (Murnane, 1945 ; Schalm and Casselberry, 1946). The experimental error of penicillin assays is very great and it is probable that it is sufficient to mask any difference in concentration less than twofold. Our estimations were made in duplicate, using both the cup and dilution methods, taking the mean of both as the reading. The considerable variation in unitage could not be related to the volume of milk, the age of the cow, the stage of lactation or the chloride content. Statistically significant differences were, however, observed between the unitage of penicillin in quarters infected with *Str. agalactiae* and quarters in completely normal animals, more being present in the latter. A greater concentration of penicillin was also recorded 12 hours and 24 hours after injection if the dose was administered in only 1 ml. of water instead of 50 ml. This result may have been due to slower diffusion through the gland or to less tissue reaction. When the larger volume was used no assessable penicillin was present in the gland after 24 hours in 50 per cent. of the cows and it is, therefore, probable that a dose of 20,000 units is too low for the best therapeutic results. On several occasions an abrupt drop in milk yield occurred after the injection. This did not result, as would be anticipated, in an increase in the concentration of penicillin but in a decrease. Attempts to determine the cause were not successful. It could not be related to the batch of penicillin, the diluent or the temperature of the injection.

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